ANALYSIS OF THE ASSEMBLY OF *SACCHAROMYCES CEREVISIAE* CYTOCHROME *C* OXIDASE SUBUNIT COX2 BY THE OXA1 AND COX18 TRANSLOCASES

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ANALYSIS OF THE ASSEMBLY OF *SACCHAROMYCES CEREVISIAE* CYTOCHROME *C* OXIDASE SUBUNIT COX2 BY THE OXA1 AND COX18 TRANSLOCASES

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Oxa1 and Cox18 are members of a conserved protein family of integral inner membrane translocases responsible for insertion of other proteins into the membranes of chloroplasts, bacteria and mitochondria. In *Saccharomyces cerevisiae* both Oxa1 and Cox18 are required for the translocation of the hydrophilic domains of cytochrome *c* oxidase subunit Cox2 into the inner membrane space. Deletions in either *oxa1* or *cox18* result in yeast strains unable to respire. Over-expression of *OXA1* does not allow for respiration in strains with deletions in *cox18,* but does allow for export of the C-terminus of Cox2. This suggests that Cox18 also has a role in the assembly of Cox2 into functional cytochrome *c* oxidase following the translocation of the C-terminus. I identified a physical interaction between Cox18 and the non-canonical chaperone Cox20, which is dependent on the presence of the Cox2 peptide. I isolated spontaneous respiring pseudorevertants from *cox18* deletion strains carrying *OXA1* on a high copy plasmid. Several of these pseudorevertants have mutations in residue 291 of the plasmid borne Oxa1. Substitution of charged residues, both positive and negative, at position 291 results in *OXA1* alleles that can compensate for a deletion in *cox18* when present in high copy number or when expressed from the

chromosomal locus. Neutral substitutions at residue 291 of Oxa1 result in alleles that fail to compliment *cox18* when present in high copy. These results show that the presence of a charged residue at position 291 allows Oxa1 to assemble Cox2 independent of Cox18 and its partner Mss2. The mutant alleles all retain Oxa1 function to various degrees. In addition, the alleles with charged residues compensate for a *mss2* deletion. Cox20 is not required for assembly by these mutant proteins, although Cox20's chaperoning of Cox2 processing is required. Therefore, these mutant alleles are not simply replacing Cox18 in its normal complexes. Rather, they may interact with the Cox2 peptide in a novel way that makes it competent for assembly.

BIOGRAPHICAL SKETCH

Leah Elliott was born in Royal Oak, MI in 1984. At the age of 7, she moved with her family to upstate New York, where they still reside. In elementary school, Leah decided that she wanted to be a teacher when she grew up, a goal that she maintains to this day. She became intrigued by biology until 7th grade when she modeled predator-prey relationships using dried beans. Leah discovered scientific research and poster sessions in her sophomore year of high school, under the guidance of Mrs. Mary Colvard and Mrs. Susan McConnelee. Although she did not succeeded in extending the lifespan of albino corn plants through the delivery of glucose in a hydroponic solution, she gained an appreciation for the scientific method and the challenges that come with any research project.

Leah stayed close to home and attended Skidmore College in Saratoga Springs, NY. While there, she conducted research with Dr. David Domozych on the fresh water desmid, *Pleurotaenium trabecula*, identifying novel cell wall structures and studying how the algae were involved in biofilm formation. This project introduced her to microscopy and the joy of discovery that come with scientific research. In the summer of 2005, Leah did an undergraduate research experience in Dr. Maureen Hanson's lab at Cornell University. During this summer, Leah helped to identify sites of RNA editing in the *Arabidopsis thaliana* mitochondrial genome under the guidance of Dr. Stéphane Bentolila. Leah graduated *summa cum laude* from Skidmore College in 2006 with a B.A. in Biology.

After graduation, Leah spent a year teaching English as a second language to

elementary and junior high school students in Shibukawa City, Japan. Leah has been interested in Japan for almost as long as she has been in biology. Not only was this year a chance to authentically experience Japan, but to experience teaching separated from biology.

Leah returned to the USA in August 2007, where she immediately started her graduate studies at Cornell University, in the Field of Genetics and Development. Continuing her interest in plant biology, she rotated in Dr. Jian Hua and worked with the bonsai *Arabidopsis* mutants. Deciding to try a different system in a lab that was studying fascinating biological puzzles, Leah rotated in Dr. Tom Fox's lab, where she decided to stay for her doctoral work.

Dedicated to the family, friends and mentors whose love, support, and encouragement allowed me to reach this.

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I would also like to thank my other committee members. Dr. Maureen Hanson gave me a wonderful introduction into Cornell and this allowed me to decide that there was nowhere else I wanted to do my PhD. I have always been grateful to her for directing my application to the Department of Molecular Biology and Genetics, where I have found an academic home. Dr. Jeff Pleiss always had a novel approach or technique to suggest, pushing me to consider a high-throughput view that our lab generally lacks.

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Before coming to Cornell University, I had many mentors who supported my interest in biology, research and learning in general. My heartfelt thanks go to Mrs. Mary Colvard and Mrs. Susan McConnelee at the Cobleskill-Richmondville High School, and Drs. David Domozych, Catherine Domozych, and Pat Hilleren at Skidmore College.

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I have been blessed with fantastic friends during my time in Ithaca. I could not have asked for better roommates than Tiffany and Warshi.; meeting you was the best thing to come out of Maplewood. Game nights at Laura and Andre's introduced me to the fun of German-style board games (made all the better with the excellent company of Ali, Satyaki, Gabe, and Mohan). One of the few regrets I have is the years it took me to realize what wonderful people and friends Christine and Courtney are. Although we are all scattering, I firmly believe that these friendships will continue, no matter how far we travel, or how long we go between meetings.

Although they are at the end of the list, my family were my first supporters. My parents, Tom Elliott and Pat O'Connor, have always given me unconditional support and love; they may not have always understood what I'm doing, but I've never doubted that they were behind me 100%. Robin is both my sister and my friend, and I am so glad we been so close these past five years.

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Chapter 1

Introduction

Mitochondria are the site of oxidative phosphorylation, which produces the ATP that acts as energy source for many cellular reactions, as well as many other biochemical pathways (ATTARDI and SCHATZ 1988). Mitochondria are believed to be derived from α-proteobacteria (LANG *et al.* 1999; GRAY *et al.* 2001). They still contain their own genomes, but during the co-evolution of the organelle and the eukaryotic cell, many genes have been lost from the mitochondria and transferred to the nucleus (reviewed in KLEINE *et al.* 2009; RICHARDS and ARCHIBALD 2011). Although it is believed that a single event resulted in a common ancestral endosymbont (GRAY *et al.* 1999), the transferred genes vary between different organisms (BURGER *et al.* 2003). For example, most mitochondrial genomes encode subunit 2 of cytochrome *c* oxidase (Cox2); however, some green algae and the legumes have transferred Cox2 into the nuclear genome (NUGENT and PALMER 1991; ADAMS *et al.* 1999).

Mitochondria are double membrane bound organelles. Starting from the cytosol, the mitochondrion is comprised of the outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and matrix (MX). The matrix contains the mitochondrial genome and its associated transcriptional and translational machinery. The inner membrane encloses the matrix, and is arranged into invaginations, termed cristae, which house the respiratory complexes. The final electron acceptor in the respiratory chain is cytochrome *c* oxidase (Complex IV). Cytochrome *c* oxidase is composed of 11 (yeast) to 13 (mammal) subunits (TSUKIHARA *et al.* 1996). The

catalytic core is formed by three subunits: Cox1, Cox2 and Cox3. These three genes are usually found in the mitochondrial genome, but the remaining peripheral subunits are encoded in the nuclear genome (reviewed (HERRMANN and FUNES 2005; FONTANESI *et al.* 2008). In addition, at least 30 additional assembly factors and chaperones are involved in inserting the subunits into the inner membrane and assembling the holoenzyme in *S. cereviaiae*. Cytochrome *c* oxidase exists as a dimer (TSUKIHARA *et al.* 1996) and associates with other complexes of the respiratory in supercomplexes (SCHAGGER and PFEIFFER 2000). Despite extensive research into the biogenesis of cytochrome *c* oxidase, there are still factors whose function or identity are unknown.

The baker's yeast *Saccharomyces cerevisiae* is a wonderful model organism for studying the mitochondria. Not only is the nuclear genome easily manipulated(GUTHRIE and FINK 1991), but the mitochondrial genome can be changed as well (BONNEFOY and FOX 2001). *Saccharomyces cerevisiae* mitochondrial genome is approximately 75kb in size and encodes eight major proteins, 24 tRNAs, 2 rRNAs and an RNA required for RNase P activity (DUJON 1981; FOURY *et al.* 1998). Therefore, most of the ~1,000 proteins that function in the mitochondria are encoded in the nuclear genome (REINDERS *et al.* 2006). All of these peptides must be imported from the cytosol into the mitochondria and sorted into the proper compartment.

There are several complexes dedicated to the import and sorting of nuclear encoded proteins into the mitochondria (reviewed in NEUPERT and HERRMANN 2007; ENDO and YAMANO 2009; WANG and DALBEY 2011; HERRMANN *et al.* 2012).

Proteins are transported first through the outer membrane by the translocase of the outer membrane (TOM) complex, which is composed of 7 subunits (KUNKELE *et al.* 1998; MODEL *et al.* 2008). There are three recognition subunits: Tom20, Tom22, and Tom70 (BRIX *et al.* 1999). The translocase pore is formed by the β-barrel Tom40 (HILL *et al.* 1998; AHTING *et al.* 2001) and stabilized by three small proteins Tom5, Tom6 and Tom7 (HONLINGER *et al.* 1996; DEMBOWSKI *et al.* 2001; BECKER *et al.* 2011); these three proteins are not absolutely required for the assembly of Tom40 into the membrane (SHERMAN *et al.* 2005).

Once in the inner membrane, proteins encounter the translocase of the inner membrane (TIM) complex. There appear to be two TIM complexes: one that inserts proteins into the matrix (Tim23 complex), and one that inserts proteins into the inner membrane (Tim22 complex). The Tim23 complex is composed of 9 subunits. The core contains the essential subunits Tim23, Tim50 and Tim17, and the nonessential Tim21 (MORO *et al.* 1999; CHACINSKA *et al.* 2005; MOKRANJAC *et al.* 2005). Tim21 contacts Tom22 in the IMS and has been suggested to coordinate movement of substrate from the TOM complex to the TIM complex (CHACINSKA *et al.* 2005; MOKRANJAC *et al.* 2005). The movement of N-terminus of the imported proteins through Tim23 is powered by the membrane potential, but to get the rest of the protein into the matrix requires a motor composed of Tim44, Tim16, Tim14, mtHsp70, and Mge1 (reviewed in NEUPERT and HERRMANN 2007). The Tim22 complex is composed of 6 subunits. Tim22, Tim54 and Tim18 are all integral membrane proteins, while Tim9, Tim10 and Tim12 are all located in the inter membrane space

(reviewed in HERRMANN *et al.* 2012). Tim22 appears to form the protein pore (SIRRENBERG *et al.* 1996) but the roles of the other subunits remains unclear.

Proteins located in the matrix can be inserted into the inner membrane by the Oxa1 translocase. Oxa1 appears to act to insert the N-termini of nuclear encoded proteins into the inner membrane (reviewed in STUART 2002). Additionally, Oxa1 co-translationally inserts mitochondrailly encoded peptides. Mitochondria contain an Oxa1 paralog, Cox18,that appears to only act in the insertion and assembly of the mitochondrially encoded Cox2 protein (reviewed in BONNEFOY *et al.* 2009). The functions of Oxa1 and Cox18 will be discussed in greater detail later.

Like mitochondria, the majority of proteins in the chloroplasts are encoded in the nuclear genome and imported into the organelle from the cytosol. In order to import these proteins, chloroplasts contain analogous complexes to mitochondria: the translocon at the outer envelope of the chloroplast (TOC complex) and the translocon at the inner envelope of the chloroplast (TIC complex) (reviewed in SMITH 2006; STRITTMATTER *et al.* 2010). There are also homologs for the Oxa1/Cox18 translocases. In chloroplasts they are Alb3, Alb4 and ATREMIS (SUNDBERG *et al.* 1997; FUNES *et al.* 2004a; BENZ *et al.* 2009).

Bacteria also need to translocate peptides through a membrane; however, in bacteria these complexes are responsible for exporting peptides, rather than importing them (DOLEZAL *et al.* 2006), reviewed in DALBEY *et al.* 2011). There are two pathways: Sec dependent and Sec independent. The Sec translocase is composed of the SecYEG and SecDFyajC complexes (ECONOMOU and WICKNER 1994; POGLIANO

and BECKWITH 1994; VEENENDAAL *et al.* 2004). The YidC translocase comprises the Sec dependent pathway (SAMUELSON *et al.* 2000; CHEN *et al.* 2002). YidC is also required by the Sec translocase for the lateral movement of transmembrane domains into the membrane (BECK *et al.* 2001). YidC is homologous to Oxa1, Cox18, Alb3, Alb4, and ATREMIS; these all form a family of translocases.

Strikingly, the Oxa1/YidC/Alb3 protein family is found across kingdoms (YEN *et al.* 2001; FUNES *et al.* 2009; FUNES *et al.* 2011). In addition to being present in bacteria and eukaryotes (both animals and plants), some euryarchaea genomes suggest the presence of members of this proteins family, although to date no functional studies have been done (YEN *et al.* 2001; POHLSCHRODER *et al.* 2005; BONNEFOY *et al.* 2009; ZHANG *et al.* 2009) All members of this family are involved in the biogenesis of large, energy transducing protein complexes (reviewed in BONNEFOY *et al.* 2009; WANG and DALBEY 2011; SALLER *et al.* 2012). Structurally, the family members fall into three groups: those like YidC, those like Oxa1 and Alb3, and those like Cox18 (FUNES *et al.* 2009) (Figure 1.1). All three groups share a conserved core structure consisting of four or five transmembrane domains, with the spacing between these domains being highly conserved (YEN *et al.* 2001; JIANG *et al.* 2003). Analysis of the single family member in *E. coli*, YidC, suggests that the structure of the core rather than the sequence of the various domains is critical for insertase activity (JIANG *et al.* 2003).

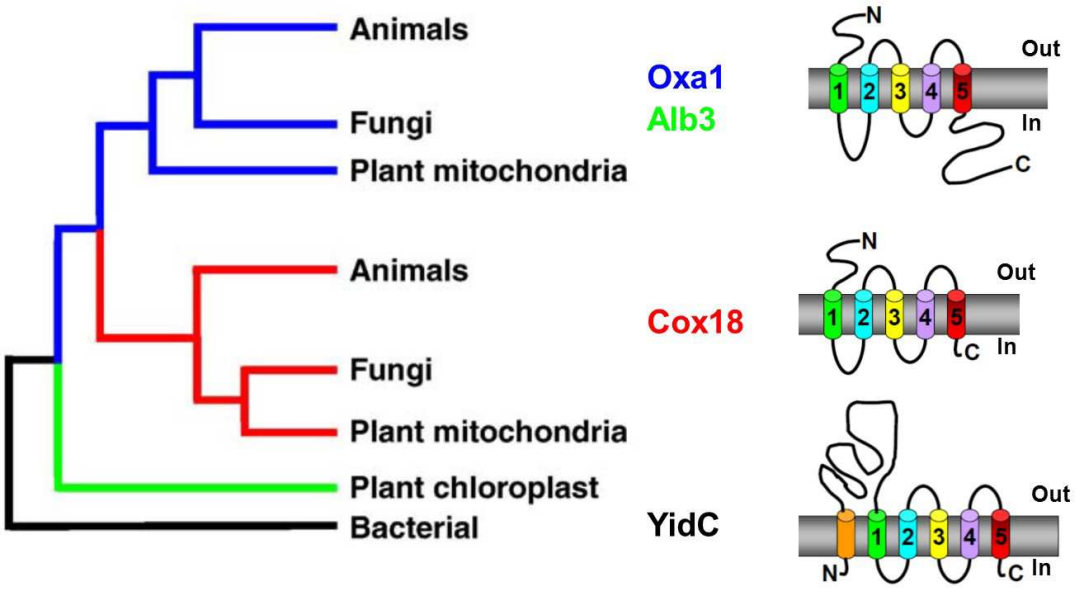


Figure 1.1* Phylogenic relationship of the Oxa1/YidC/Alb3 protein family. Each of the three structural groups is cartooned, showing the conserved core of 5 transmembrane domain as well as differences at the N- and C-terminal regions.

* Adapted from BONNEFOY, N., H. L. FIUMERA, G. DUJARDIN and T. D. FOX, 2009 Roles of Oxa1-related inner-membrane translocases in assembly of respiratory chain complexes. Biochim Biophys Acta **1793:** 60-70. and reprinted with permission

The YidC like proteins contain an extra transmembrane domain and large periplasmic loop at the N-terminus (SAAF *et al.* 1998; YEN *et al.* 2001; FUNES *et al.* 2009). However, this loop and additional transmembrane domain are dispensable (JIANG *et al.* 2003). Oxa1 like proteins contain an extension at the C-terminus after the core region (YEN *et al.* 2001; FUNES *et al.* 2009). This C-terminal region has been shown to interact with ribosomes (JIA *et al.* 2003; SZYRACH *et al.* 2003; JIA *et al.* 2009) and is required for co-translational insertion, but not post-translation insertion of substrates (SZYRACH *et al.* 2003). The third, Cox18 like group of proteins lack extensions of either termini. Across this family, proteins have a four-fold variance in length, from 225 residues to 795 residues (YEN *et al.* 2001).

Despite the structurally conserved core region, there is little conservation of primary sequence between family members; a survey of this core from 76 proteins in this family only found one fully conserved residue (YEN *et al.* 2001). However, this family has conserved functionality, even across kingdoms. For example, deletion of the *Saccharomyces cerevisiae oxa1* can be complemented by *OXA1* homologs from human (BONNEFOY *et al.* 1994), *Arabidopsis thalania* (HAMEL *et al.* 1997), and the yeasts *S. pombe* (BONNEFOY *et al.* 2000) and *N. crassa* (NARGANG *et al.* 2002). This functional conservation is all the more impressive when the specificity of each homolog is considered (reviewed in BONNEFOY *et al.* 2009). Loss of Oxa1 in *S. cerevisiae* results in complete loss of respiratory complex IV and decreased levels of complex III and V (BAUER *et al.* 1994; BONNEFOY *et al.* 1994; MEYER *et al.* 1997b; LEMAIRE *et al.* 2000; JIA *et al.* 2007). However, low levels of Oxa1 in human cultured cells causes a decrease in complex I and V, but an increase in complex IV (STIBUREK

et al. 2007) and deletion of *oxa1* in the yeast *N. crassa* causes decreased levels of complex I and IV, but no detectable changes to any other complexes (NARGANG *et al.* 2002).

Organisms encode between one and six proteins that belong to the YidC/Oxa1/Alb3 family (YEN *et al.* 2001). Most gram-negative bacteria contain a single, YidC like protein (YEN *et al.* 2001; FUNES *et al.* 2009). Mitochondria and gram-positive bacteria contain two family members, one Oxa1 like and one Cox18 like (FUNES *et al.* 2009; ZHANG *et al.* 2009; FUNES *et al.* 2011). Chloroplasts likewise contain two family members, one like Alb3 and one like Alb4 from *Arabidopsis thaliana*. Phylogenic analysis of the entire protein family suggests that there were separate gene duplication events that occurred in mitochondria, chloroplasts and gram-positive bacteria; this then suggests that the C-terminal extensions found on Oxa1 homologs and Alb3 homologs resulted from independent events (FUNES *et al.* 2009). Interestingly, organisms that have degenerate mitochondria (VAN DER GIEZEN and TOVAR 2005) or degenerate plastids (CAVALIER-SMITH 2000; MACASEV *et al.* 2000) that lack the energy transducing complexes also lack members of this protein family. It is unclear if the substrates or the Oxa1/Cox18/Alb3 proteins were lost first, or if the losses occurred simultaneously.

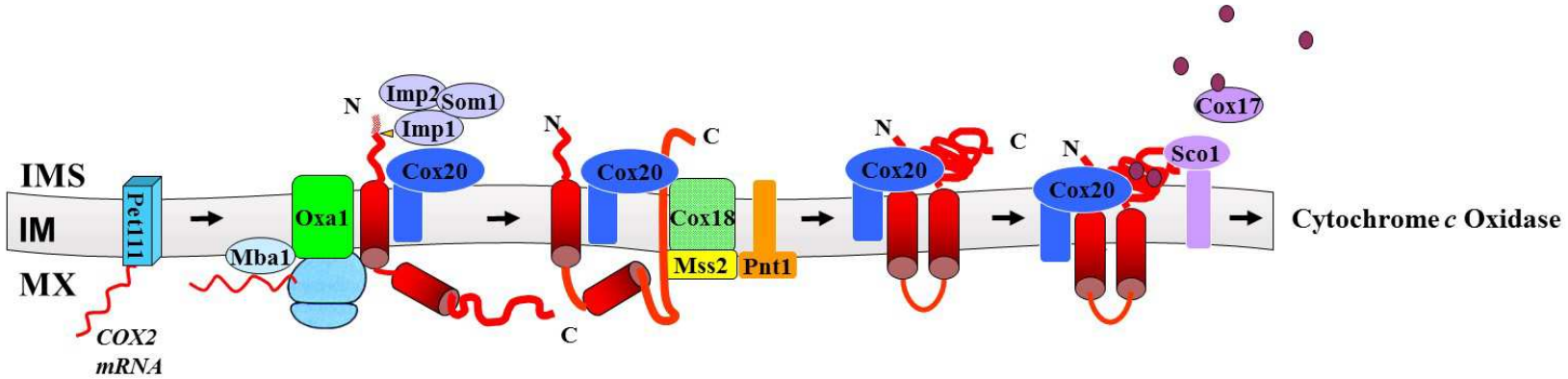
The mitochondrial paralogs Oxa1 and Cox18 have been extensively studied in *S. cerevisiae* (reviewed in BONNEFOY *et al.* 2009). These two proteins have evolved distinct and separate functions (PREUSS *et al.* 2005). Oxa1 acts to insert a variety of substrates into the inner membrane: the mitochondrially-encoded proteins Cox1,

Cox2, Cox3, Cyt*b* and Atp9 (HE and FOX 1997; HELL *et al.* 1997; HELL *et al.* 2001; JIA *et al.* 2007) and Oxa1 has been proposed to generally insert nuclear encoded peptides that have their N-termini inserted into the inner membrane (HELL *et al.* 2001; reviewed in STUART 2002), including Oxa1 itself (HERRMANN *et al.* 1997; HELL *et al.* 1998). In contrast, Cox18 appears to be responsible for the export of the C-terminus of Cox2 alone (SARACCO and FOX 2002; FIUMERA *et al.* 2007). Oxa1 interacts with the mitochondrial ribosomes through its extended C-terminal domain (JIA *et al.* 2003; SZYRACH *et al.* 2003; JIA *et al.* 2009). Cox18 has been shown to physically interact with Mss2 and Pnt1 (HE and FOX 1999; SARACCO and FOX 2002; FIUMERA *et al.* 2007) and Cox20 (ELLIOTT *et al.* 2012). In addition to insertase activity, Cox18 has a role in the assembly of Cox2 into Cytochrome *c* oxidase (FIUMERA *et al.* 2009). The interaction between Oxa1 and the ribosome has a role in the formation of supercomplexes in the respiratory chain, specifically in III_2/IV and III_2/IV_2 (KEIL *et al.* 2012).

To understand the differences between these paralogs, several domain switching experiments were conducted. Cox18 from yeast is able to complement the function of YidC in *E. coli* (VAN BLOOIS *et al.* 2007). In a reciprocal experiment, YidC from *E. coli* was shown to complement a deletion in the yeast *S. cerevisiae cox18* but not a deletion of *oxa1* (PREUSS *et al.* 2005). But when the C-terminal ribosome-binding domain from yeast Oxa1 was fused onto YidC, then YidC complemented a deletion of *oxa1* but could no longer complement a deletion of *cox18* (PREUSS *et al.* 2005). This suggests that the ribosome-binding domain of Oxa1 is essential for its function and inhibits Cox18 function.

Despite the differences between them, both Oxa1 and Cox18 act on the same substrate, subunit 2 of cytochrome *c* oxidase. However, they are just two of almost a dozen nuclear encoded factors that are known to be required for the production, insertion and assembly of the mitochondrially encoded Cox2 peptide in *S. cerevisiae* (reviewed in FONTANESI *et al.* 2006) (Figure 1.2). The Cox2 mRNA is transcribed from the mitochondrial genome in the matrix. The mRNA is localized to the matrix side of the inner membrane by the translational activator Pet111 (POUTRE and FOX 1987; MULERO and FOX 1993; SANCHIRICO *et al.* 1998; BONNEFOY *et al.* 2001; GREEN-WILLMS *et al.* 2001; NAITHANI *et al.* 2003) which binds the 5` untranslated leader sequence (GREEN-WILLMS *et al.* 2001; NAITHANI *et al.* 2003). Proper localization of the Cox2 messenger is required for productive assembly of the Cox2 peptide (SANCHIRICO *et al.* 1998). In fact, Pet111 is present at levels in the cell that make it the rate limiting step of Cox2 expression (GREEN-WILLMS *et al.* 2001). Oxa1 co-translationally inserts the N-terminus of Cox2 through the inner membrane (HE and FOX 1997; HELL *et al.* 1997). Mba1, an inner membrane protein which also interacts with the mitochondrial ribosome, is also required for N-terminal insertion (PREUSS *et al.* 2001; OTT *et al.* 2006).

Figure 1.2 Biogenesis of subunit 2 of cytochrome *c* oxidase (Cox2). See text for descriptions of each protein's function. IMS = intermembrane space, IM = inner membrane, MX = matrix, mt = mitochondrial


IMS
IM
MX
Pet111
Mba1
Oxa1
Imp2
Som1
Imp1
Cox20
Cox18
Mss2
Pnt1
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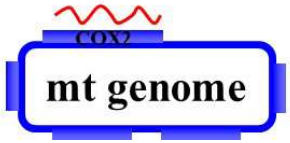
Sco1
N
C
COX2
mRNA
Cytochrome c Oxidase


COX2
mt genome

Yeast Cox2 is produced as a precursor; once the N-terminus is inserted and present in the intermembrane space, the first 15 amino acids are cleaved off by Imp1, a part of the inner membrane protease complex (SEVARINO and POYTON 1980; PRATJE *et al.* 1983; NUNNARI *et al.* 1993). Other components of this protease complex include Imp2 and Som1 (NUNNARI *et al.* 1993; ESSER *et al.* 1996; JAN *et al.* 2000). Cleavage of this leader peptide, to produce a mature Cox2 peptide, is chaperoned and enhanced by the non-canonical chaperone Cox20 (HELL *et al.* 2000). The human FAM36A protein is an ortholog of Cox20 but cannot be involved in processing Cox2 as the mammalian Cox2 peptide is synthesized without this leader sequence (SZKLARCZYK *et al.* 2013). Cleavage of this leader peptide is likely required for respiration; mutation of the cleavage site results in a respiratory defect in otherwise wild type cells (SARACCO 2003; PEREZ-MARTINEZ *et al.* 2009). However, there is a report of a mutation in *COX2* that allows it to bypass the need for Oxa1 and for this processing event (MEYER *et al.* 1997a).

After the Cox2 peptide is fully translated, the C-terminus is also inserted through the inner membrane, a step that requires a number of factors. Oxa1 is also required for the export of the C-terminus, although this may reflect a need for N-terminal export prior to C-terminal export (HE and FOX 1997). Cox18 is the translocase responsible for moving the C-terminus through the membrane (SARACCO and FOX 2002). Cox18 physically associates with Mss2 and Pnt1 on the matrix side of the inner membrane (SARACCO and FOX 2002). Mss2 is peripherally associated with the matrix side of the inner membrane (BROADLEY *et al.* 2001). Deletion of *mss2* causes an accumulation of Cox2 that has the N-terminus exported into the

intermembrane space, but retains the C-terminus in the matrix (BROADLEY *et al.* 2001). Mss2 is hypothesized to recognize the Cox2 C-terminus after translation and help to bring it to Cox18 for translocation (BROADLEY *et al.* 2001; FIUMERA *et al.* 2007). Pnt1 is bound in the inner membrane with both termini present in the matrix (HE and FOX 1999). Deletion of *pnt1* has only a modest effect on respiratory growth in *S. cerevisiae*, although deletion of the *Kluyuveromyces lactis* homolog results in a respiratory defect (HE and FOX 1999). Previous work on *cox20* deletion strains hinted at a Cox2 C-tail export defect (HELL *et al.* 2000). There is a requirement for Cox20 in the export of the Cox2 C-tail (HELL *et al.* 2000; SARACCO 2003; ELLIOTT *et al.* 2012)and a physical interaction between Cox20 and the Cox18 translocase (ELLIOTT *et al.* 2012).

After both termini are present in the intermembrane space, copper is inserted into the Cu_A site present in the C-terminus of Cox2. Copper ions are bound by the copper chaperone Cox17 (ABAJIAN *et al.* 2004; HORNG *et al.* 2004) (BEERS *et al.* 1997; ABAJIAN *et al.* 2004; HORNG *et al.* 2004). The insertion of these ions is then facilitated by the chaperone Sco1 (HORNG *et al.* 2004). Sco1 co-immunoprecipitates with Cox2, independent of copper binding (LODE *et al.* 2000). Although *in vitro* passing of copper from Cox17 to Sco1 can be observed (HORNG *et al.* 2004) it has yet to be demonstrated *in vivo*. Mammals contain a Sco1 homolog, Sco2, which is also required for copper insertion into the Cu_A site and for Cox2 protein synthesis (LEARY *et al.* 2009). The biochemical mechanism of Sco2 in Cox2 synthesis has not yet been elucidated. In copper insertion, it is possible that Sco2 acts to keep the cysteine residues in Sco1 reduced (CHINENOV 2000; BALATRI *et al.* 2003; LEARY *et al.* 2009).

When any of the steps above are disrupted, the unassembled Cox2 peptide is degraded by several proteases. When the Cox2 peptide has both termini inserted into the inner membrane, but fails to be incorporated into sub-complexes or the cytochrome *c* oxidase holoenzyme, it is degraded by Yme1, the ATP dependent metallopeptidase of the inter membrane space (*i*-AAA) (NAKAI *et al.* 1995; WEBER *et al.* 1996). Two accessory proteins, Mgr1 and Mgr3, associate with Yme1 and are thought to recognize and deliver substrates to the protease (DUNN *et al.* 2008). When C-terminal export is blocked, the majority of Cox2 peptide is degraded by the AAA protease of the matrix (*m*-AAA) (BROADLEY *et al.* 2001). The *m*-AAA protease is a hetro-oligomer composed of Yta10 and Yta12 (ARLT *et al.* 1996; LEE *et al.* 2011). Interestingly, both the *i*- and *m*-AAA proteases appear to have chaperone functions that are independent of their proteolytic abilities (ARLT *et al.* 1996; GRAEF and LANGER 2006; GRAEF *et al.* 2007; FIUMERA *et al.* 2009; FRANCIS and THORSNESS 2011).

Assembly of Cox2 into sub-complexes and the holoenzyme is not well understood. Four assembly intermediate sub-complexes (S1-S4) of Cytochrome *c* oxidase were identified in human cells (NIJTMANS *et al.* 1998). Cox2 only appeared in the latter two stages (S3 and S4). This model was further refined to eight intermediate sub-complexes using cells from patients with mutations in either *SCO2* or *SURF1*, factors required for cytochrome *c* oxidase formation (STIBUREK *et al.* 2005). However, Cox1 was the only subunit of the holoenzyme detected in these additional, smaller sub-complexes. The assembly factors and chaperones involved in the formation of these complexes, in general, have yet to be described. The Cox20

chaperone is likely involved, as all the mature Cox2 appears to be bound to Cox20 when assembly of cytochrome *c* oxidase is disrupted in *S. cerevisiae* by deletion of the nuclear component *cox4* (HELL *et al.* 2000; MCSTAY *et al.* 2013).

Cox18 also has a role in assembling Cox2 after its C-terminus is translocated into the intermembrane space (FIUMERA *et al.* 2009). Fiumera *et al.* (2009) found that when *cox18* was deleted in *S. cerevisiae* and its paralog *OXA1* was overexpressed, Cox2 had both termini present in the intermembrane space. However, this strain fails to respire, indicating that although Cox2 was inserted into the membrane, it was not being assembled into cytochrome *c* oxidase. Interestingly, disruption of either Yme1 accessory protein, Mgr1 or Mgr3, allowed for slow respiratory growth in this *cox18* deletion, *OXA1* overexpression strain; respiration in this case required the chaperone function of Yme1 (FIUMERA *et al.* 2009). It is thought that in the absence of Cox18, high levels of Oxa1 are capable of translocating both termini into the intermembrane space, but in a way that renders them unable to be assembled into productive cytochrome *c* oxidase. Normally, this unassembled Cox2 is recognized by either Mrg1 or Mgr3 and brought to Yme1 as a substrate for degradation. However, in the absence of these accessory proteins, Yme1 interacts with the unassembled Cox2 as a chaperone, allowing it to become assembled into cytochrome *c* oxidase. This is the first reported bypass of Cox18's function; this dissertation focuses on additional bypass mechanisms.

In this dissertation, I show that Cox18 physically interacts with the non-canonical chaperone Cox20, and that this interaction is dependent on the presence of

the Cox2 peptide. I further show that disrupting the *i*-AAA protease complex in the absence of Cox20 restores N-terminal processing and respiratory growth; therefore Cox20 is not absolutely required for either function. I also report on mutations in codon 291 of Oxa1 that allow Oxa1 to compensate for a deletion in its paralog *cox18*. The ability of these alleles to restore respiratory growth in a *cox18Δ* background do not depend the normal Cox2 insertion machinery; Cox20 is required, but apparently only for N-terminal processing.

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Chapter 2

Multiple Roles of the Cox20 Chaperone in Assembly of *Saccharomyces cerevisiae* Cytochrome *c* Oxidase[1]

Introduction

Cytochrome *c* oxidase is composed of three subunits encoded in the mitochondrial genome (mtDNA) and eight subunits encoded in the nuclear genome in the budding yeast *Saccharomyces cerevisiae*. In addition to the genes for these subunits, at least 20 other nuclear yeast genes are specifically required for synthesis of the mitochondrially coded subunits and post-translational steps in assembly of the active enzyme (BARRIENTOS *et al.* 2002; HERRMANN and FUNES 2005; FONTANESI *et al.* 2006).

The second largest subunit of yeast cytochrome *c* oxidase, Cox2, is a mitochondrial gene product whose acidic N-terminal and C-terminal domains are translocated through the inner membrane from the matrix to the intermembrane space (IMS) and flank two transmembrane helices (TSUKIHARA *et al.* 1996). Cox2 topogenesis is of particular interest since its hydrophilic domains are the largest

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known to be exported through the inner membrane. In budding yeast, localized membrane-bound translation of the *COX2* mRNA, specifically activated by Pet111 (GREEN-WILLMS *et al.* 2001; NAITHANI *et al.* 2003) produces a precursor, pre-Cox2, with a short N-terminal leader peptide (PRATJE *et al.* 1983). The pre-Cox2 N-tail is co-translationally exported by Oxa1 (HE and FOX 1997; HELL *et al.* 1998), a highly conserved inner membrane translocase that is also required for C-tail export (reviewed in BONNEFOY *et al.* 2009). Once in the IMS, the pre-Cox2 leader peptide is rapidly processed by the inner membrane protease (IMP) (NUNNARI *et al.* 1993; JAN *et al.* 2000), in a reaction chaperoned by Cox20 (HELL *et al.* 2000).

The acidic Cox2 C-tail is exported to the IMS by a mechanism that is distinct from N-tail export and appears to be post-translational (HE and FOX 1997; FIUMERA *et al.* 2007). C-tail export depends specifically upon another highly conserved inner membrane translocase, Cox18 (SARACCO and FOX 2002), which is paralogously related to Oxa1, as well as to bacterial YidC proteins (FUNES *et al.* 2004b). In addition, Cox2 C-tail export requires Mss2 and is promoted by Pnt1, two proteins that interact with Cox18 (HE and FOX 1999; BROADLEY *et al.* 2001; SARACCO and FOX 2002). Interestingly, overproduction of Oxa1 in a mutant lacking Cox18 results in some export of the Cox2 C-tail, although Cox2 remains unassembled and the cells fail to respire (FIUMERA *et al.* 2009). This result suggested that Cox18 has an assembly function in the IMS that overproduced Oxa1 cannot carry out, in addition to its translocation function. One possibility here is that Cox18 could promote interaction of the exported Cox2 C-tail with an assembly factor in the IMS. One candidate for such a factor is Cox20.

Cox20 has previously been shown to be a 205-amino-acid integral mitochondrial inner membrane protein. It has two centrally located transmembrane helices flanked by hydrophilic domains in the intermembrane space (HELL *et al.* 2000). Cox20 interacts directly with pre-Cox2 and promotes its processing. Since this interaction depends upon export of pre-Cox2 by Oxa1, it appears to involve domains in the IMS (HELL *et al.* 2000). Cox20 is also critical for export of the C-terminus of Cox2. In mitoplasts derived from wild type cells, a C-terminally tagged Cox2-HA variant is exposed on the outer surface of the inner membrane and the tag is degraded by the addition of protease (SARACCO and FOX 2002; FIUMERA *et al.* 2007). In addition to its roles in processing and export, Cox20 remains associated with unassembled mature Cox2, suggesting that it has roles in cytochrome *c* oxidase assembly downstream of pre-Cox2 processing (HELL *et al.* 2000; PREUSS *et al.* 2001; HERRMANN and FUNES 2005), possibly including Cox2 metallation (RIGBY *et al.* 2008). Cox20 has therefore been described as a chaperone, although it has no detectable similarity to other well-characterized chaperones or domains of known function.

In this study I have investigated the role of Cox20 in the export and assembly of Cox2. I find that Cox20 is required for efficient export of the Cox2 C-tail and that it interacts with the translocase Cox18 but only when Cox2 is present. In addition, Cox20 stabilizes mature but unassembled Cox2.

Materials and Methods

Yeast strains and genetic analysis of pseudorevertants

S. cerevisiae strains used in this study are listed in Table 2.1. All strains are congenic to D273-10B (ATCC 25657). Nuclear genes were manipulated using standard methods (GUTHRIE and FINK 1991). Transformation of plasmids and PCR products into yeast was accomplished with the EZ transformation kit (Zymo Research). Complete media (YPA) containing adenine, dextrose (D), ethanol plus glycerol (EG), or raffinose (R) were prepared as previously described (GUTHRIE and FINK 1991). Complete synthetic media (CSM) and CSM lacking specific growth factors were purchased from Bio101 Systems. *COX20* was modified to encode a protein tagged with three MYC epitope at its C terminus, but with no other changes, by the pop-in pop-out strategy as described (SCHNEIDER *et al.* 1995). The plasmid pOXA1-W56R-ADH1 (SUPEKOVA *et al.* 2010) was obtained from F. Supek and P. G. Schultz.

Table 2.1

Strains and plasmids used in this study

Name	Nuclear (mitochondrial) genotype	Reference
3590	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 cox20Δ::KanMX4 (ρ^+)*	Open Biosystems
CAB116	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 (ρ^+)*	This study

Name	Nuclear (mitochondrial) genotype	Reference
DFS188	*MATa ura3-52 leu2-3,112 lys2 his3*Δ *arg8*::*hisG* (ρ^+)	Steele *et al.* (1996)
LEE80	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG COX20::3xMYC* (ρ^+)	This study
LEE83	*MATα lys2 ade2 cox20Δ::URA3* (ρ^+)	This study
LEE92	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII COX20::3xMYC COX18::3xHA* ($\rho+$)	This study
LEE112	*MATa ura3-52 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG COX20::3xMYC COX18::3xHA* ($\rho+$ *cox2-20*)	This study
LEE98	*MATα leu2-3,112 lys2 his3ΔHinDIII cox20Δ::URA3 mgr3Δ::KanMX4* (ρ^+)	This study
LEE99	*MATα lys2 his3ΔHinDIII cox20Δ::URA3 mrg1Δ::KanMX4* (ρ^+)	This study
LEE100	*MAT a ura3Δ lys2 arg8::hisG ade2 cox20Δ::URA3 mgr3Δ::KanMX4* (ρ^+)	This study

Name	Nuclear (mitochondrial) genotype	Reference
LEE121	*MATa ura3-52 leu2-3,112 lys3 his3ΔHinDIII arg8::hisG pOXA1-W56R-ADH1* (ρ^+ *cox2-10)*	This study
LEE122	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pOXA1-W56R-ADH1* (ρ^+)	This study
LEE123	*MATα ura3 ade2 oxa1Δ::LEU2 pOXA1-W56R-ADH1* (ρ^+)	This study
LEE126	*MATa ura3-52 leu2-3,112 pet111-9 pOXA1-W56R-ADH1* (ρ^+)	This study
LEE127	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 cox20Δ::KanMX49 pOXA1-W56R-ADH1* (ρ^+)	This study
LEE128	*MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG imp1Δ::KanR pOXA1-W56R-ADH1* (ρ^+)	This study
LEE139	*MATa leu2-3,112 lys2 arg8::hisG ade2 trp1-1 cox20Δ::URA3 yme1Δ::URA3 imp1Δ::KanR* (ρ^+)	This study

Name	Nuclear (mitochondrial) genotype	Reference
NB40-3C	*MATa ura3-52 leu2-3,112 lys2 his3DHinDIII arg8::hisG (r+ cox2-62)*	Bonnefoy and Fox (2000)
NB60	*MATa ura3-52 leu2-3,112 lys3 his3ΔHinDIII arg8::hisG (ρ^+ cox2-10)*	Bonnefoy and Fox (2000)
NB151-9B	*MATa ura3-52 leu2-3,112 pet111-9 (ρ^+)*	This study
pOXA1-W56R-ADH1	*COX2-W56R URA3*	Supekova et al. (2010)
SCS101	*MATa ura3-2 leu2-3,112 lys2 his3Δ arg8::hisG (ρ+ COX2::3xHA)*	This study
SCS114	*MATa ura3-53 leu2-3,112 his3Δ arg8::hisG (ρ+ cox20N15::3xHAI)*	This study
SCS182	*MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG cox20Δ::URA3 (ρ+ COX2::3xHA)*	This study
SCS192	*MATa ura3-52 leu3-3,112 lys2 his3Δ arg8::hisG (ρ+ COX2::3xHA cox3Δ::ARG8^m)*	This study
SCS193	*MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG imp1Δ::KanR (ρ^+)*	Fiori *et al* (2003)

Name	Nuclear (mitochondrial) genotype	Reference
SCS218	*MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG (ρ+ COX2::3xHA cox1Δ::ARG8^m)*	This study
NB40-3C	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG (ρ$^+$ cox2-62)*	Bonnefoy and Fox (2000)

All strains congenic to D273-10B, except 3590 and LEE127 which are congenic to BY4741

Independent spontaneous pseudorevertants of the *cox20Δ* strain LEE83 were isolated by plating cells from distinct clonal cultures on YPAEG plates and incubating at 30° for 7 to 10 days. A single pseudorevertant was picked from each clonal culture, purified, and mated to *cox20Δ* (SCS194), *cox20Δmgr1Δ* (LEE145), *cox20Δmgr3Δ* (LEE100), and *cox20Δyme1Δ* (LEE106). The resulting diploids were isolated and their ability to grow on YPAEG was assessed to determine that the mutations were recessive and to score their ability to complement the known genes.

Analysis of mitochondrial proteins

To examine export of the C terminus of Cox2, strains whose mtDNA encodes a version of Cox2 bearing C-terminal HA-epitopes were employed (SARACCO and FOX 2002). Mitoplasts were prepared by osmotic shock from purified mitochondria as described (GLICK 1995; GLICK and PON 1995). For each sample of mitoplasts, the equivalent of 75 μg of mitochondrial protein was treated with 20 μg/ml proteinase K,

or mock treated, as described (SARACCO and FOX 2002), except that after protease treatment samples were directly resuspended in 20 mM HEPES pH 7.4, 0.6 M sorbitol, 10% trichloroacetic acid, and 2 mM PMSF and incubated at 60° for 10 min.

For co-immune precipitation experiments, crude mitochondria were prepared as described (DIEKERT *et al.* 2001). One milligram of mitochondrial protein was solubilized in 1 ml of 1% digitonin, 100 mM NaCl, 20 mM Tris pH 7.4, and 1 mM PMSF for 10 min on ice and then centrifuged at 16,000 × g for 10 min at 4°. Five percent of the clarified supernatant was precipitated using Strataclean resin (StrataGene). The remaining supernatant was incubated with anti-hemagglutinin (anti-HA) antibody conjugated beads (Roche). After incubation for 1 hr at 4°, the beads were washed three times in the digitonin buffer before proteins were eluted in 5× Laemmli SDS sample buffer.

Protein samples to be analyzed by Western blotting were separated on 12% or 16% SDS-PAGE gels as indicated and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore). Antibodies were anti-HA monoclonal antibody clone 3F10 (Roche) (except the experiment of Figure 1, where anti-HA-HRP conjugate (Roche) was used), anti-c-myc (anti-Myc) monoclonal antibody clone 9E10 (Roche), polyclonal anti- Cit1 (citrate synthase), polyclonal anti-Yme1, and monoclonal anti-Cox2 CCO6. Anti-mouse IgG-HRP (BioRad) or anti-rabbit IgG-HRP (BioRad) secondary antibodies were applied after washing and detected by the ECL and ECL+ detection methods (GE Healthcare).

In vivo pulse-labeling of mitochondrial translation products was carried out as described (BONNEFOY *et al.* 2001) in cells grown on YPAR medium, except that there was no chase incubation, and mitochondria were prepared as described (DIEKERT *et al.* 2001). Radiolabeled mitochondrial proteins were separated on 16% SDS PAGE gels, which were dried and autoradiographed.

Results

Cox20 is required for Cox2 C-tail export independently of cytochrome c oxidase assembly or Cox2 N-tail processing

Cox20 is known to be required for the N-terminal processing of pre-Cox2 and to remain associated with mature Cox2 prior to its assembly into cytochrome c oxidase (HELL *et al.* 2000). Before Cox2 can be assembled, the C-tail must be translocated from the matrix to the intermembrane space. As a part of his dissertation work, Scott Saracco asked whether Cox20 plays a role in the export of the Cox2 C-tail (SARACCO 2003). He purified mitochondria from strains whose mtDNA encodes a variant of Cox2 with three HA-epitope tags at its C terminus and then converted to mitoplasts lacking the outer membrane. This epitope is accessible to protease added to wild-type mitoplasts, whose Cox2 C-tail has been translocated through the inner membrane (SARACCO and FOX 2002; FIUMERA *et al.* 2007) (Figure 1). Protease digestion of mitoplasts from a *cox20Δ* strain did not destroy the C-terminal epitope, but did shorten the tagged protein by digestion of the exported Cox2 N-tail domain; the C-tail epitope was digested by protease when the mitoplasts were solubilized by the addition of the mild detergent octyl glucoside (Figure 1). Thus, Cox20 is required for export of the

Cox2 C-tail domain through the inner membrane, but not for the mechanistically distinct (HE and FOX 1997) N-tail export process.

Hell *et al.* (HELL *et al.* 2000) had previously observed a defect in Cox2 C-tail export in the *cox20Δ* mutant. However, they attributed this effect to decreased inner membrane potential caused by the lack of cytochrome c oxidase in the mutant. Scott Saracco investigated this possibility further by examining the topology of the HA epitope in strains lacking either Cox1 or Cox3, the other two core subunits of cytochrome c oxidase. In both cases the C-terminal epitope on Cox2 was accessible to protease in mitoplasts from the mutants (Figure 1), confirming that Cox2 C-tail export is not prevented indirectly by the absence of cytochrome c oxidase activity (SARACCO and FOX 2002).

Since Cox20 is required for N-terminal processing of the pre-Cox2 leader peptide, Scott Saracco next asked whether this processing is required for C-tail export. Pre-Cox2 is processed by cleavage after residue 15 by the Imp1 subunit of the inner membrane protease complex (PRATJE *et al.* 1983; BEHRENS *et al.* 1991; NUNNARI *et al.* 1993). Neither the cox2-N15I processing site mutation(SARACCO 2003; PEREZ-MARTINEZ *et al.* 2009) nor the *imp1Δ* mutation, both of which prevent pre-Cox2 leader peptide processing, prevented Cox2 C-tail export as measured by protease sensitivity of the Cox2 C-terminal HA epitopes in mitoplasts (Figure 1). (While some HA-reactive Cox2 was detected in the *imp1Δ* mitoplasts treated with protease, this is largely attributable to inefficient mitoplasting, as evidenced by the partial protection of the IMS marker Yme1.)

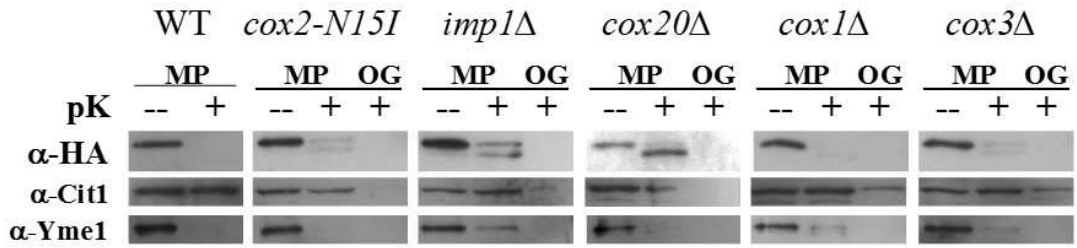


Figure 2.1 Export of the C terminus of pre-Cox2 is dependent on Cox20, but not on N-terminal processing or cytochrome c oxidase assembly.* Mitoplasts (MP) from strains expressing Cox2 with a C-terminal HA tag were either mock treated or treated with 20mg/ml of proteinase K (pK) (Materials and Methods) as indicated. Samples were solubilized with 1% octyl glucoside (OG) prior to protease treatment where indicated. For wild type, 5 mg of proteins were loaded per lane, and for the mutants, 50 mg of protein were loaded per lane. The resulting blots were probed with a-HA, a-Cit1, and a-Yme1 antibodies and developed with the ECL detection system (Invitrogen). Strains are indicated as follows: WT (SCS101), *cox2-N15I* (SCS114), *imp1Δ* (SCS184), *cox20Δ* (SCS182), *cox1Δ* (SCS218), and *cox3Δ* (SCS192).

* This experiment was carried out by S.A. Saracco. The figure is adapted from SARACCO 2003.

Taken together, these data show that Cox20 has a role in the export of the Cox2 C-tail that is independent of the requirements for Cox20 in pre-Cox2 processing and cytochrome c oxidase assembly.

Cox20 interacts dynamically with Cox18 in a Cox2-dependent manner

Cox18 is the inner membrane translocase responsible for moving the Cox2 C-tail into the intermembrane space (SARACCO and FOX 2002; BONNEFOY *et al.* 2009). To further investigate the role of Cox20 in translocation, I tested for physical interaction between Cox18 and Cox20 by co-immune precipitation. Mitochondria were isolated from cells containing epitope-tagged Cox20-Myc and either tagged Cox18-HA or wild-type Cox18. Digitonin solubilized extracts were immunoprecipitated with anti-HA antibody bound to agarose beads, and the precipitates were analyzed by Western blotting (Materials and Methods). Cox20–Myc coprecipitated with Cox18–HA but was not precipitated from extracts containing untagged Cox18 (Figure 2).

Cox20 and Cox18 could either interact in a stable complex or could interact dynamically during the translocation of the Cox2 C-tail. To distinguish between these possibilities I asked whether Cox20–Myc and Cox18–HA co-immune precipitation depends on the presence of Cox2. Cox2 translation was prevented by introducing the *cox2-20* mutation (TORELLO *et al.* 1997; BONNEFOY *et al.* 2001) into the mtDNA of a strain containing Cox20–Myc and Cox18–HA. A mitochondrial extract from this strain was analyzed as described above, revealing that the interaction between Cox20

and Cox18 was disrupted when Cox2 synthesis was blocked (Figure 2). Thus, mitochondrially coded Cox2 appears to bridge the interaction between the translocase Cox18 and the chaperone-like protein Cox20.

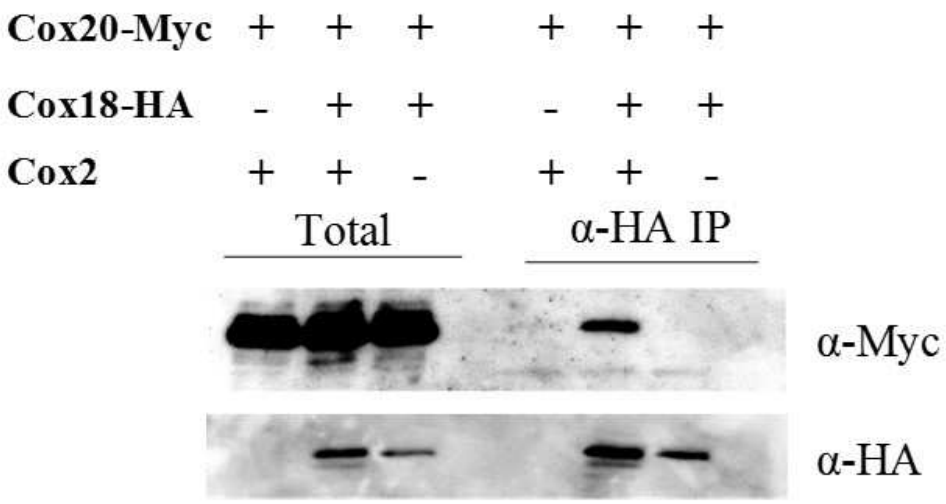


Figure 2.2 Co-immune precipitation of Cox20–Myc with Cox18–HA is dependent on the presence of Cox2. Mitochondria were prepared from strains LEE80 (*COX20::Myc*), LEE92 (*COX20::Myc COX18::HA*), and LEE112 (*COX20::Myc COX18::HA* [*cox2-20*]), solubilized in buffer containing 1% digitonin, and subjected to immune precipitation with anti-HA beads (Materials and Methods). Five percent of total protein from each extract (Total), and the immune precipitates (a-HA IP) were subjected to SDS–PAGE on a 12% acrylamide gel and Western blotting. The blots were decorated with anti-Myc antibody, stripped, and then redecorated with anti-HA antibody. The presence or absence of Cox20–Myc, Cox18–HA, and Cox2 proteins in the extracts are indicated by + and -.

The absence of Cox20 can be partially bypassed by disruption of the i-AAA protease complex

Cox20 is required for respiratory growth (Figure 3,(HELL *et al.* 2000). However, *cox20Δ* mutant cells plated on nonfermentable medium yielded spontaneous weakly respiring pseudorevertants. I suspected that these mutations might affect the activity of the *i*-AAA protease, on the basis of a previous study of mutations that allow overproduced Oxa1 to partially suppress a *cox18Δ* mutation (FIUMERA *et al.* 2009). Since Cox20 and Cox18 appear to function together, I tested the phenotype of double mutants containing the *cox20Δ* mutation and *mgr1Δ*, *mgr3Δ*, or *yme1Δ* (Figure 3). In a *cox20Δ* background, deletion of *YME1*, *MGR1*, or *MGR3* caused weak respiratory growth (Figure 3). However, this weak respiratory growth was dependent upon *IMP1*.

I found that 36 independent spontaneous *cox20Δ* pseudorevertants (Materials and Methods) contained recessive nuclear mutations, since they yielded nonrespiring diploids when mated with an otherwise wild-type *cox20Δ* strain. Twenty-three of these pseudorevertants appear to be due to *mgr3* mutations since they produced respiring diploids when mated to *cox20Δ mgr3Δ* strain, indicating failure to complement, but nonrespiring diploids when mated to either *cox20Δ mgr1Δ* or *cox20Δ yme1Δ* strains. Tetrad analysis confirmed that five of these pseudorevertants were indeed caused by mutations tightly linked to *mgr3Δ*. Ten of the remaining spontaneous pseudorevertants failed to complement after being mated with a *cox20Δ mgr1Δ*. The remaining three pseudorevertants produced nonrespiring diploids when mated to *cox20Δ* double mutant strains containing *yme1Δ, mgr1Δ, and mgr3Δ* mutations. The spontaneous

mutations in these three pseudorevertants failed to complement each other and were linked to each other, suggesting that they affected a single unidentified additional gene. However, these strains have been lost. This screen was apparently not saturated, since none of the spontaneous pseudorevertants contained mutations that failed to complement *yme1Δ*.

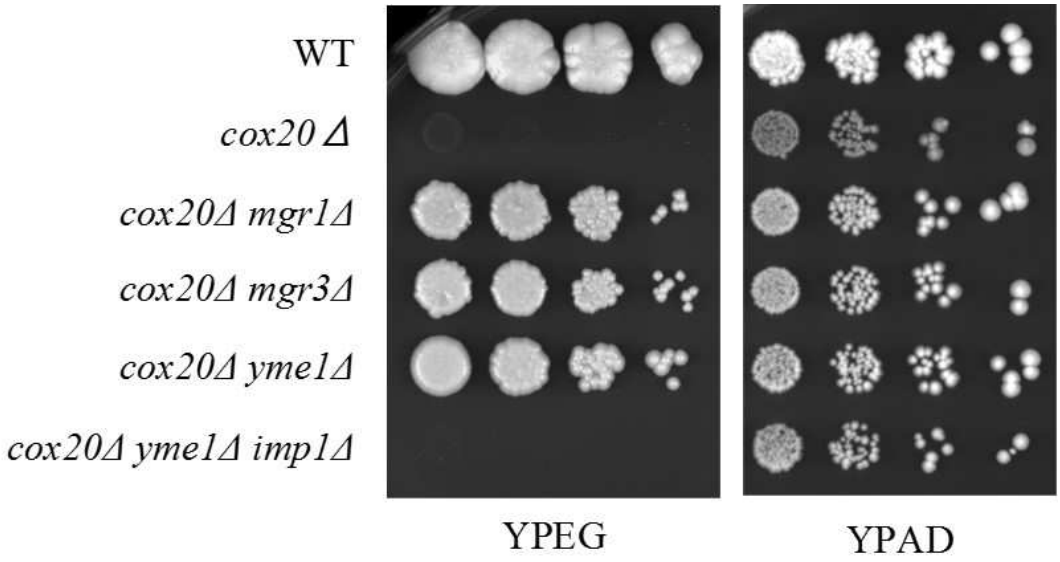


Figure 2.3 Loss of i-AAA protease activity due to yme1, mgr1, or mgr3 mutations allows weak respiratory growth of cox20Δ cells. Cells grown in rich glucose medium were diluted and spotted onto YPAEG (Materials and Methods) nonfermentable ethanol plus glycerol medium (Eth + Gly), incubated at 30_ for 14 days, spotted onto YPAD (Materials and Methods) glucose (Gluc), and incubated at 30_ for 4 days. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), *cox20Δmgr1Δ* (LEE99), *cox20Δmgr3Δ* (LEE98), *cox20Δyme1Δ* (LEE105), and *cox20Δyme1Δimp1Δ* (LEE139).

Imp1-dependent processing of pre-Cox2 in cox20Δ mutants occurs in the absence of i-AAA protease activity

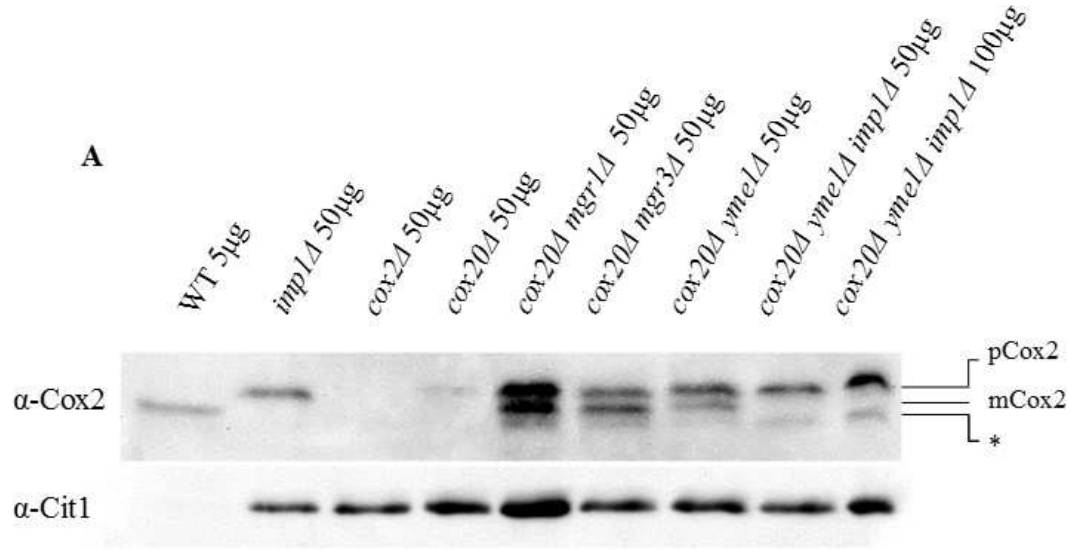
Processing of the pre-Cox2 N-terminal leader peptide is essential for assembly of cytochrome c oxidase, since mitochondrial mutations that alter the processing site cause a nonrespiratory growth phenotype in strains with wild-type nuclear genomes(BONNEFOY *et al.* 2001; SARACCO 2003; PEREZ-MARTINEZ *et al.* 2009). The fact that one function of Cox20 is to assist in this Imp1-dependent processing event (HELL *et al.* 2000) raised the question of how the pre-Cox2 N terminus is processed in *cox20Δ* stains lacking *i*-AAA protease activity. I first confirmed that mature Cox2 does accumulate at steady state in the weakly respiring *cox20Δ mgr1Δ*, *cox20Δ mgr3Δ*, and *cox20Δ yme1Δ* double mutants by Western blotting of mitochondrial proteins probed with an anti-Cox2 antibody. While processing was much less efficient in the double mutants than in wild type, each of the weakly respiring double mutants contained significant amounts of mature Cox2 (Figure 4A). The generation of mature Cox2 under these conditions is still dependent upon Imp1 activity since a *cox20Δ yme1Δ imp1Δ* triple mutant, which has a nonrespiratory growth phenotype (Figure 3), accumulated the same pre-Cox2 species as an *imp1Δ* single mutant (Figure 4A).

All of the strains deficient in *i*-AAA protease activity also accumulated detectable amounts of a smaller Cox2 species (indicated by * in Figure 4A). Since the *i*-AAA protease is known to be responsible for degradation of unassembled Cox2 (NAKAI *et al.* 1995; PEARCE and SHERMAN 1995; WEBER *et al.* 1996), the smaller Cox2 species appears to be a breakdown product of pre-Cox2 that accumulates in the absence of *i*-AAA activity. Interestingly, the *cox20Δ* mutant accumulated substantially

less pre-Cox2 than either the imp1Δ mutant or any of the *cox20Δ* strains deficient in *i*-AAA protease (Figure 4). These data suggest that Cox20 directly stabilizes pre-Cox2 against degradation by the *i*-AAA protease. Alternatively, pre-Cox2 whose C-tail has not been exported may be more labile due to its aberrant topology.

In contrast to the accumulation of immunologically detectable mature Cox2 in weakly respiring *cox20Δ* mutants deficient in *i*-AAA protease, processing of pre-Cox2 in the absence of Cox20 was not observed during 35S-pulse labeling of mitochondrial translation products in the same strains (Figure 4B). Thus, while Imp1-dependent processing of pre-Cox2 occurs in the double mutants (Figure 4A), it is too slow to be detectable during pulse labeling. These data are consistent with the proposal that Cox20 serves as a chaperone to present pre-Cox2 to the inner membrane protease complex, but is not a component of that complex (HELL *et al.* 2000).

Figure 2.4 Pre-Cox2 is processed by Imp1 in the absence of i-AAA protease activity, but inefficiently. (A) Crude mitochondrial protein samples (DIEKERT *et al.* 2001) in the amounts indicated were separated on 16% SDS–PAGE and Western blotted. The blot was decorated with both antibody specific to the C-tail of Cox2 and antibody specific against Cit1, as a loading control. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), cox2Δ (NB40-3C), *cox20Δ mgr1Δ* (LEE99), *cox20Δ mgr3Δ* (LEE98), *cox20Δ yme1Δ* (LEE105), and *cox20Δ yme1Δ imp1Δ* (LEE139). pCox2 indicates pre-Cox2; mCox2 indicates mature Cox2; * indicates a novel Cox2 cross-reacting species. (B) Cells were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide for 20 min at 30° (Materials and Methods). Mitochondria were analyzed by SDS–PAGE (16% acrylamide gel) and autoradiography. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), *cox2Δ* (NB40-3C), *cox20Δ mgr1Δ* (LEE99), *cox20Δ mgr3Δ* (LEE98), *cox20Δ yme1*Δ (LEE105), and *cox20Δ yme1Δ imp1Δ* (LEE139). The identities of major mitochondrial translation products are indicated.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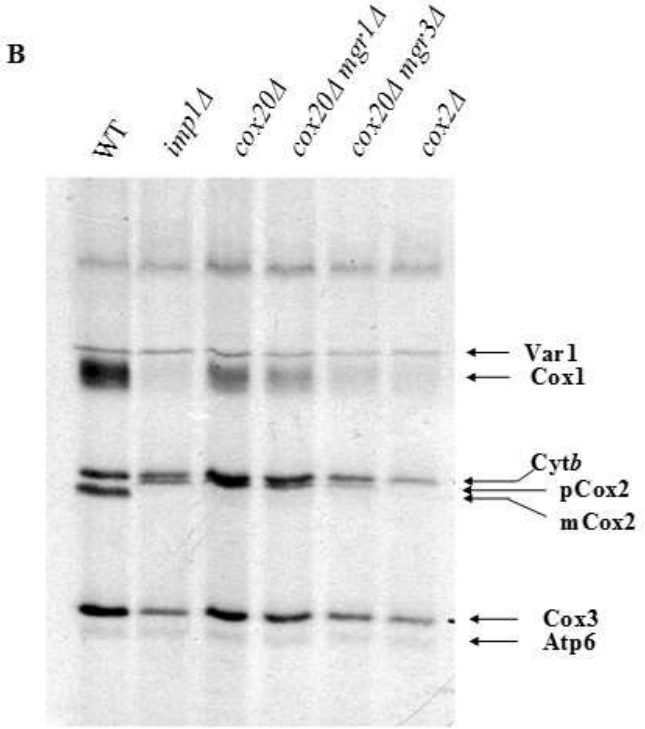
imp1Δ 50µg
cox2Δ 50µg
cox20Δ 50µg
cox20Δ mgr1Δ 50µg
cox20Δ mgr3Δ 50µg
cox20Δ yme1Δ 50µg
cox20Δ yme1Δ imp1Δ 50µg
cox20Δ yme1Δ imp1Δ 100µg
α-Cox2
α-Cit1
pCox2
mCox2
*


B
WT
imp1Δ
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cox2Δ
Var1
Cox1
Cytb
pCox2
mCox2
Cox3
Atp6

Import of a variant form of pre-Cox2 from the cytoplasm does not bypass the requirements for Cox20 and Imp1 in cytochrome c oxidase biogenesis

I attempted to dissect further the activities of Cox20 using a plasmid-borne mutant version of the *COX2* gene, that had been recoded for expression from the nucleus. This recoded *COX2* gene (denoted N-COX2 in Figure 5), specifying the amino acid substitution W56R and the mitochondrial targeting signal of the Oxa1 protein, was obtained on a high-copy plasmid termed pOXA1–W56R–ADH1 (SUPEKOVA *et al.* 2010). I transformed this plasmid into the nuclei of mutant strains (Figure 5). As expected, it strongly complemented the respiratory growth defect of a *cox2Δ* mutation in mtDNA. Furthermore, import of the nuclearly encoded, cytoplasmically synthesized variant of Cox2 also complemented the respiratory defect of a nuclear *pet111Δ* mutant that is specifically unable to translate the mitochondrial *COX2* mRNA (Figure 5). In addition, import of this protein bypassed the requirement for the Cox2 C-tail translocase Cox18, suggesting that the assembly pathway for the imported variant of Cox2 does not involve import into the matrix and subsequent reexport of the C-tail. However, import of this protein in the absence of Cox20, Imp1, or Oxa1 did not promote respiratory growth (Figure 5) or the assembly of active cytochrome c oxidase as assayed by reduction of tetramethyl-p-phenylenediamine (MCEWEN *et al.* 1985) data not shown).

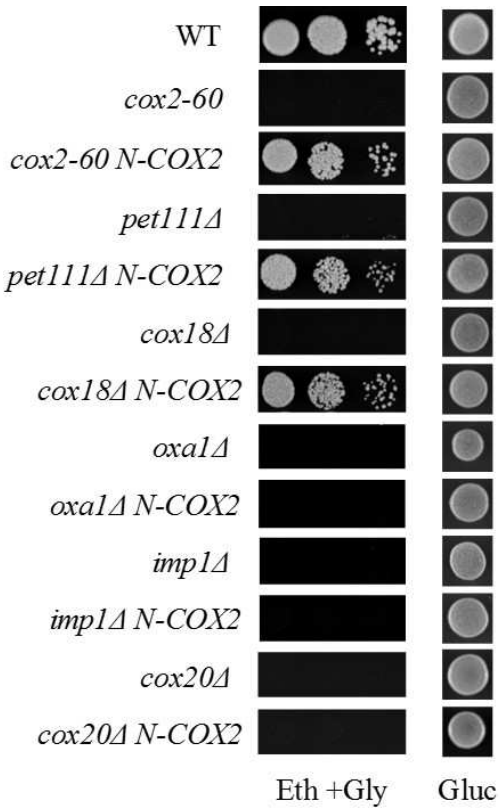

Figure 2.5 Expression of a recoded nuclear COX2 gene bypasses the requirement for Cox18 but not Cox20. Cells containing the plasmid pOXA1-W56R-ADH1 (Supekova et al. 2010) were grown in CSM–Ura, and untransformed cells were grown in CSM. Cultures were diluted and spotted onto YPAEG nonfermentable (Eth + Gly) plates and incubated at 30° for 4 days or spotted onto YPAD fermentable (Gluc) plates and incubated at 30° for 2 days. Strains are indicated as follows: WT (DFS188), *cox2-60* (NB60), *cox2-60 N-COX2* (LEE121), *pet111Δ* (NB151-9B), *pet111Δ N-COX2* (LEE126), *cox18Δ* (CAB116), *cox18Δ N-COX2* (LEE122), *oxa1Δ* (MCC319), *oxa1Δ N-COX2* (LEE123), imp1Δ (SCS193), *imp1Δ N-COX2* (LEE128), *cox20Δ* (LEE173), *cox20Δ N-COX2* (LEE174).

Discussion

The experiments presented here argue strongly that Cox20 has an important role in Cox2 C-tail topogenesis, in addition to its previously established function in pre-Cox2 leader peptide processing. First, in the absence of Cox20, mitochondrially synthesized unassembled pre-Cox2 accumulates with its N-tail domain in the IMS and its C-tail domain in the matrix, showing that Cox20 is required for efficient C-tail translocation through the inner membrane. Second, this defect in Cox2 C-tail export cannot be due to the defect in pre-Cox2 processing caused by the absence of Cox20, since C-tail export was not affected by either *cox2* or *imp1* mutations, which also prevent pre-Cox2 processing. Finally, Cox20 co-immune precipitates with the Cox2 C-tail-specific translocator Cox18 from mitochondria synthesizing pre-Cox2, but not from mitochondria lacking a functional COX2 gene. Thus, the mitochondrial gene product Cox2 appears to bridge a dynamic interaction between Cox20 and Cox18.

These data are consistent with the findings of Hell *et al*. (HELL *et al.* 2000) who reported that a significant fraction of the Cox2 C-tail, newly synthesized in isolated *cox20Δ* mitochondria, remained on the matrix side of the inner membrane after pulse labeling, while the N-tail was efficiently exported. Our findings that the vast majority of Cox2 molecules accumulated in a *cox20Δ* mutant have an N-out, C-in topology, while both N- and C-tails of Cox2 are efficiently exported in other mutants that lack cytochrome c oxidase activity, argue that the export defect in *cox20Δ* mitochondria is not a secondary effect due to reduced membrane potential, as previously suggested (HELL *et al.* 2000).

On the basis of these results, I propose that Cox20 binding to Cox2 accelerates dissociation of newly exported Cox2 from the Cox18 translocase on the IMS side of the inner membrane. Thus, while not directly involved in membrane translocation *per se*, Cox20 is required for efficient cycling of the Cox18 translocase.

Unassembled Cox2 is largely degraded by the *i*-AAA protease Yme1 (NAKAI *et al.* 1995; PEARCE and SHERMAN 1995; WEBER *et al.* 1996), which is bound to the inner membrane facing the IMS (LEONHARD *et al.* 1996). Yme1 is associated with two other proteins, Mgr1 and Mgr3, that recognize and deliver at least some substrates for degradation by Yme1 (DUNN *et al.* 2006; DUNN *et al.* 2008). A study of interspecies Yme1 chimeras comprising combinations of homologous domains of the *S. cerevisiae* and *Neurospora crassa* proteins identified Cox20 as a factor influencing the pathways by which unassembled Cox2 substrate could reach the *i*-AAA proteolytic domain (GRAEF *et al.* 2007).

The results presented in this Chapter strongly suggest that a third critical chaperone function of Cox20 is to protect as-yet-unassembled Cox2 from degradation by the *i*-AAA protease complex during the assembly process downstream of export. Furthermore, they indicate a strong dependence of Cox2 degradation upon the putative substrate recognition factors Mgr1 and Mgr3, at least in the absence of Cox20. In cells lacking Cox20, the steady-state level of accumulated pre-Cox2 was extremely low, and those cells exhibited a tight nonrespiratory phenotype. However, in cells lacking Cox20 as well as Yme1, Mgr1, or Mgr3, the steady-state levels of pre-Cox2 and mature Cox2 were increased, and those cells exhibited weak respiratory growth

reflecting the assembly of low levels of cytochrome c oxidase. Consistent with these findings, Hell et al. (HELL *et al.* 2000) observed that virtually all residual unassembled pre-Cox2 or mature Cox2 was associated with Cox20 when cytochrome c oxidase assembly was blocked by either an *imp1Δ* or a *cox4Δ* mutation, respectively. The C-terminal domain of Cox20 is basic, which may facilitate its interaction with the acidic N- and C-tails of Cox2 following their export to the IMS.

Cox20 is required for normal rates of pre-Cox2 processing by the Imp1 subunit of the inner membrane protease. However, slow Imp1-dependent processing does occur in the absence of Cox20 if pre-Cox2 is stabilized by loss of the *i*-AAA protease. In contrast, the requirement for Imp1 is not bypassed by elimination of the *i*-AAA protease.

Although Cox20 is also required for normal export of the Cox2 C-tail domain, low levels of export and assembly into cytochrome *c* oxidase are detectable in the absence of Cox20 if *i*-AAA activity is also absent. On the basis of the model that Cox20 promotes dissociation of exported Cox2 C-tail from the Cox18 translocase, I suggest that in the absence of Cox20 the *i*-AAA protease rapidly degrades unprotected Cox2 C-tail that is associated with Cox18. In the absence of both Cox20 and the *i*-AAA protease, slow dissociation of exported Cox2 from Cox18 can occur and leads to limited cytochrome c oxidase assembly.

Supekova et al. (SUPEKOVA *et al.* 2010) recently described a recoded and modified version of the *COX2* gene that supports respiratory growth when allotopically expressed from the yeast nucleus in a *cox2Δ* mutant. As expected, this

gene, termed here *N-COX2*, bypassed the requirement for Pet111, which normally activates translation of the *COX2* mRNA inside mitochondria. Interestingly, it also bypassed the requirement for the Cox2 C-tail-specific export translocase Cox18. This finding suggests that the cytoplasmically synthesized C-tail domain of allotopically expressed pre-Cox2 remains in the intermembrane space during import into mitochondria, rendering Cox18 superfluous. However, *N-COX2* did not bypass the requirement for Cox20. Since expression of *N-COX2* required Imp1, Cox20 is presumably required to chaperone the cleavage of imported pre-Cox2 by the inner membrane protease. Cox20 may also be required for stabilization of unassembled imported Cox2, and possibly chaperoning its further assembly.

Previous work in this laboratory has shown that overproduced Oxa1 can partially bypass the requirement for Cox18 in export of the Cox2 C-tail domain, but not the requirement for Cox18 in cytochrome c oxidase assembly (FIUMERA *et al.* 2009). This observation suggested that in addition to translocating the Cox2 C-tail through the inner membrane, Cox18 normally delivers the C-tail in a fashion promoting assembly, possibly by interacting with downstream assembly factors. This study supports this idea, and indicates that Cox20 may be an assembly factor that can acquire the exported Cox2 C-tail from Cox18, but not from overproduced Oxa1. (On the basis of the experiments with *N-COX2*, I postulate that Cox20 can interact with the Cox2 C-tail imported from the cytoplasm in the absence of Cox18.) Interestingly, *mgr1Δ* or *mgr3Δ* mutations, but not *yme1Δ*, allowed overproduced Oxa1 to promote weak cytochrome c oxidase assembly and respiratory growth in the absence of Cox18. These findings suggested that in the absence of the substrate recognition factors Mgr1

and/or Mgr3, the AAA+ protein Yme1 could function as a chaperone for Oxa1-exported Cox2, instead of degrading it (Fiumera et al. 2009). In light of these results, it is tempting to speculate that the role of Cox20 vis-à-vis the Cox18 translocase is carried out by latent Yme1 chaperone activity when the Cox2 C-tail is aberrantly exported by overproduced Oxa1.

Cox18 is highly conserved. Indeed the homologous proteins from humans (GAISNE and BONNEFOY 2006), fission yeast (GAISNE and BONNEFOY 2006), *N. crassa* (FUNES *et al.* 2004b), and *E. coli* (PREUSS *et al.* 2005) can partially complement a *cox18Δ* in *S. cerevisiae* at the level of respiratory growth. A Cox20 homolog (NCBI NP_932342.1) has also been identified in humans (HERRMANN and FUNES 2005). A mammalian ortholog of Cox20 could not have a role in Cox2 processing since mammalian Cox2 proteins are not synthesized as precursors (STEFFENS and BUSE 1979; ANDERSON *et al.* 1982). However, a mammalian Cox20 protein could participate with Cox18 in Cox2 C-tail export as well as Cox2 stabilization and assembly into cytochrome *c* oxidase.

Recently a human patient homozygous for a mutant allele of the human *COX20* ortholog, *FAM36A*, was identified (SZKLARCZYK *et al.* 2013). Biochemical analysis of fibroblasts derived from this patient showed decreased levels of the FAM36A protein, decreased levels of cytochrome *c* oxidase subunits, and decreased levels and activity of cytochrome *c* oxidase. Assembly of cytochrome *c* oxidase was disrupted at the steps when Cox2 is normally incorporated (NIJTMANS *et al.* 1998; SZKLARCZYK *et al.* 2013). Szklarczyk *et al* (SZKLARCZYK *et al.* 2013) also showed

that wild type FAM36A specifically co-immunprecipitates with Cox2. Taken together, it appears that the role for Cox20 in the assembly of Cox2 into cytochrome *c* oxidase truly is conserved.

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Chapter 3

Substitutions in the 4th transmembrane domain of the Oxa1 translocase allow it compensate for the loss of its paralog Cox18 and to assemble Cox2 in *Saccharomyces cerevisiae*

Introduction

Cytochrome *c* oxidase is the terminal acceptor of the electron transport chain. The catalytic core of this enzyme is composed of three large subunits, Cox1, Cox2 and Cox3, which are encoded in the mitochondrial genome in yeast and mammals. This core is surrounded by nuclear encoded peptides and requires many additional nuclear encoded factors, at least 30 in *Saccharomyces cerevisiae*, for the synthesis and assembly of the core peptides (reviewed in FONTANESI *et al.* 2006; FONTANESI *et al.* 2008; MCSTAY *et al.* 2013). The Cox2 subunit alone requires about a dozen factors: Pet111 for translational activation of the *COX2* mRNA; Oxa1 for co-translational insertion of the N-terminus; the inner membrane protease complex (Imp1, Imp2 and Som1) with Cox20 for cleavage of 15 residues from the N-terminus; the Cox18 translocase, in conjunction with Mss2 and Pnt1, for insertion of the C-terminus through the inner membrane and Cox20 to release the inserted peptide from the translocase; and Sco1 and Cox17 for the insertion of copper into the Cu_A site in the C-terminal domain.

Oxa1 and Cox18 are paralogs and members of a conserved family of translocases. Other family members include YidC in bacteria and the Albino proteins in chloroplasts. Members of this family insert subunits of energy-transducing and respiratory complexes via membrane insertase and protein tranlocase activities (reviewed in BONNEFOY *et al.* 2009; SALLER *et al.* 2012). Translocases from this family share a core topology of five transmembrane domains. Only Oxa1 homologs contain a C-terminal extension that is required for interactions with the mitochondrial ribosome (JIA *et al.* 2003; SZYRACH *et al.* 2003; JIA *et al.* 2009). Homologs are functionally conserved across kingdoms: deletion of the yeast *oxa1* can be complemented by *OXA1* genes from human and the flowering plant *Arabidopsis thaliana* (BONNEFOY *et al.* 1994; HAMEL *et al.* 1997). *cox18* deletions can similarly be complemented by the human homolog and by the *E. coli* family member YidC (GAISNE and BONNEFOY 2006; VAN BLOOIS *et al.* 2007). YidC from *E. coli* can complement a deletion in the yeast *S. cerevisiae cox18* but not a deletion of *oxa1* (PREUSS *et al.* 2005). Fusing the C-terminal ribosome-binding domain from yeast Oxa1 onto YidC, allows YidC to complement a deletion of *oxa1* but not a deletion of *cox18* (PREUSS *et al.* 2005). This suggests that the ribosome-binding domain of Oxa1 is essential for its function and inhibits Cox18 function.

Although Oxa1 and Cox18 are paralogs, they have distinct roles in the biogenesis of Cox2 in *S. cerevisiae*. Oxa1 is required to insert the N-terminus (HE and FOX 1997); this is thought to occur co-translationally. Oxa1 is also required for C-terminal export, but it is still unclear if this is due to a requirement for sequential insertion of the termini or due to a direct action by Oxa1 (HE and FOX 1997;

BONNEFOY *et al.* 2009) . Conversely, Cox18 only has one known substrate, Cox2. Cox18 is not required for N-terminal export of Cox2, but is required for C-terminal export, along with Mss2 and Pnt1 (HE and FOX 1997; BROADLEY *et al.* 2001; SARACCO and FOX 2002). Cox18 also interacts with the non-canonical chaperone Cox20, which is thought to help disassociate the fully inserted Cox2 peptide from the Cox18 translocase (ELLIOTT *et al.* 2012).

In the absence of *cox18*, overexpression of *OXA1* fails to restore respiratory growth in *S. cerevisiae*, but does result in Cox2 peptide with both termini translocated into the intermembrane space (SARACCO and FOX 2002; FIUMERA *et al.* 2009). This is evidence that in addition to its function as a translocase, Cox18 also has a role in the assembly of Cox2. Although overexpression of *OXA1* from a high copy plasmid fails to compensate for deletion of *cox18* at the level of growth, spontaneous respiring pseudorevertants do occur, and a class of nuclear recessive suppressors have already been described (FIUMERA *et al.* 2009). An additional class of plasmid-linked pseudorevertants can be isolated. Here I report on substitutions at residue 291of Oxa1 that allow for respiratory growth in the absence of *cox18*. These mutant Oxa1 proteins promote the assembly of Cox2 in a novel way, as they do not make Oxa1 more similar to Cox18 at the level of primary sequence, nor do they require Cox18's normal interactors Mss2, Pnt1 and Cox20 to assemble Cox2 into functional cytochrome *c* oxidase.

Materials and Methods

Yeast strains and genetic analysis of pseudorevertants

S. cerevisiae strains used in this study are listed in Table 3.1. Nuclear genes were manipulated using standard methods (GUTHRIE and FINK 1991). Transformation of plasmids and linear DNA into yeast was accomplished with the EZ transformation kit (Zymo Research). Complete media (YPA) containing adenine, dextrose (D), ethanol plus glycerol (EG), or raffinose (R).were prepared as previously described (GUTHRIE and FINK 1991). Complete synthetic media (CSM) and CSM lacking specific growth factors were purchased from Bio101 Systems.

Table 3.1

Strains and plasmids used in this study

All strains are congenic to D273-10B

Name	Nuclear (mitochondrial) genotype	Reference
1c sup rho+	*MATα uar3-52 leu2-3,112 lys2 his3Δ arg8::hisG cox18Δ::URA3 pLE6 (ρ^+)*	This study
CAB116	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 (ρ^+)*	Elliott *et al.* (2012)
DFS188	*MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG (ρ^+)*	Steele *et al.* (1996)

Name	Nuclear (mitochondrial) genotype	Reference
LEE41	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 (ρ^+)*	This study
LEE43	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 oxa1Δ::URA3 pLE6 (ρ^+)*	This study
LEE44	*MATα ura3Δ::KanMX leu2-3,112 his4-59 OXA1-T291K (ρ^+)*	This study
LEE47	*MATα ura3-52 leu2-3,112 arg8::hisG mss2Δ::URA3 pLE6 (ρ^+)*	This study
LEE55	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 oxa1Δ::URA3 pLE6 (ρ^+)*	This study
LEE81	*MATa ura3-52 or ura3Δ leu2-3,112 lys2 his3ΔHinDIII cox18Δ::KanMX cox20Δ::URA3 (ρ^+)*	This study
LEE96	*MATa ura3-52 or ura3Δ leu2-3,112 lys2 his3ΔHinDIII cox18Δ::KanMX cox20Δ::URA3 pLE6 (ρ^+)*	This study
LEE99	*MATα lys2 his3ΔHinDIII cox20Δ::URA3 mrg1Δ::KanMX4 (ρ^+)*	Elliott *et al.* (2012)

Name	Nuclear (mitochondrial) genotype	Reference
LEE156	*MATa ura3-52 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox20Δ::URA3 pLE6 (ρ^+)*	This study
LEE171A	*MATa ura3Δ lys2 his3ΔHinDIII arg8::hisG cox18Δ::URA3 cox20Δ::URA3 mgr1Δ::KanMX4 (ρ^+)*	This study
LEE172A	*MATa ura3Δ lys2 his3ΔHinDIII arg8::hisG cox18Δ::URA3 cox20Δ::URA3 mgr1Δ::KanMX4 pLE6 (ρ^+)*	This study
LEE181	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pLE6 (ρ^+)*	This study
LEE182	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pLE9 (ρ^+)*	This study
LEE183	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pLE12 (ρ^+)*	This study
LEE185	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pLE14 (ρ^+)*	This study
LEE186	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pEL15 (ρ^+)*	This study

Name	Nuclear (mitochondrial) genotype	Reference
LEE196	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 pNB189 (ρ^+)*	This study
LEE198	*MATa ura3-52 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::URA3 OXA1-T291K (ρ^+)*	This study
LEE199	*MATa ura3-25 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox18Δ::KanMX4 oxa1Δ::URA3 pLE6 (ρ^+)*	This study
LEE200	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pNB189 (ρ^+)*	This study
LEE201	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pLE6 (ρ^+)*	This study
LEE202	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pLE9 (ρ^+)*	This study
LEE203	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pLE12 (ρ^+)*	This study
LEE204	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pLE13 (ρ^+)*	This study
LEE205	*MATα ura3Δ::KanMX leu2-3,112 his4-59 oxa1Δ::URA3 pLE14 (ρ^+)*	This study

Name	Nuclear (mitochondrial) genotype	Reference
MCC319	*MATα ura3 ade2 oxa1Δ::LEU2 (ρ^+)*	Elliott *et al.* (2012)
SCS59	*MATα uar3-52 lue2-3,112 lys2 his3Δ arg8::hisG cox18Δ::URA3 (ρ^+)*	Saracco (2003)
SCS194	*MATa ura3-52 leu2-3,112 lys2 his3ΔHinDIII arg8::hisG cox20Δ::URA3 (ρ^+)*	Elliott *et al.* (2012)
XPM184a	*MATα ura3-52 leu2-3,112 lys2 arg8::hisG mss2Δ::URA3 (ρ^+)*	SHINGÚ-VÁZQUEZ *et al.* (2010)
pLE6	*OXA1-T291K LEU2*	This study
pLE9	*OXA1-T291R LEU2*	This study
pLE12	*OXA1-T291E LEU2*	This study
pLE13	*OXA1-T291H LEU2*	This study
pLE14	*OXA1-T291P LEU2*	This study
pLE15	*OXA1-T291A LEU2*	This study
pNB189	*OXA1 LEU2*	Gaisne and Bonnefoy (2006)

Independent spontaneous pseudorevertants of the diploid *cox18Δ/cox18Δ [OXA1]* strain LEE40 were isolated by plating cells from distinct clonal cultures on YPAEG plates and incubating at 30° for 21days. A single pseudorevertant was picked from each of the three clonal cultures that gave rise to them, and purified. Plasmids were lost from pseudorevertants by growing overnight in non-selective YPAD at 30°C then streaked for single colonies onto YPAD. These colonies were replica printed to YPAEG and CSM lacking leucine (CSM-Leu) and the resulting patterns analyzed.

Plasmids from the pseudorevertants were prepared from yeast strains as previously described (SHERMAN *et al.* 1986). Plasmids were then transformed into DH5-α *E. coli* (Invitrogen) per manufacturer's instructions and isolated again using a Qiagen mini-prep kit. The plasmids isolated from *E. coli* isolation were sequenced to determine the sequence of the *OXA1* gene present on the plasmid.

Plasmids used in this study were created from pNB189 by using site-directed mutagenesis (QuickChange® II from Stratagene) to change codon 291 of the *OXA1* gene. All primers for these plasmids were similar, with only the sequence corresponding to codon 291 (underlined in the primer sequences) changing. pLE6/T291K was produced using primers 040809A (CTATACCGGCCAAAATGAACTTATCG) and 040809B (CGATAAGTTCATTTTGGCCGGTATAG). pLE9/T291R was produced using primers 040809C (CTATACCGGCCAGAATGAACTTATCG) and 040809D (CGATAAGTTCATTCTGGCCGGTATAG). pLE12/T291E was produced using primers 052009A (CTATACCGGCCGAAATGAACTTATCG) and 052009B (CGATAAGTTCATTTCGGCCGGTATAG). pLE13/T291H was produced using

primers 070209C (CTATACCGGCCCACATGAACTTATCG) and 070209D (CGATAAGTTCATGTGGGCCGGTATAG). pLE14/T291P was produced using primers 070209A (CTATACCGGCCCCAATGAACTTATCG) and 070209B (CGATAAGTTCATTGGGGCCGGTATAG). pLE15/T291A was produced using primers 082809A (CTATACCGGCCGCAATGAACTTATCG) and 082809B (CGATAAGTTCATTGCGGCCGGTATAG).

OXA1-T291K was integrated into the chromosomal locus by amplifying from plasmid pLE6 with Phusion high-fidelity polymerase (NEB) and using primers 111208A (GGAAGTAATCCGAGCCATGAA) and 111208B (CAAGAAGACTTAGCCAACAGC). The resulting linear DNA was used to transform the *oxa1Δ::URA3* yeast strain LEE41 such that integration removed *URA3*. Candidates were selected on 5FOA, and integration was verified by PCR and sequencing. The resulting *OXA1-T291K COX18* strain (LEE44) was crossed to *oxa1Δ::URA3 cox18Δ::KanMX4* strain LEE43 and the tetrads were dissected to generate *OXA1-T291K cox18Δ::KanMX4* strain LEE198.

Analysis of mitochondrial proteins

To examine the topology of Oxa1-T291K in the inner membrane, mitoplasts were prepared by osmotic shock from purified mitochondria of cells grown in CSM plus raffinose or CSM-leucine plus raffinose as described (GLICK and PON 1995). For each sample of mitoplasts, the equivalent of 100 μg of mitochondrial protein was treated with 20 μg/ml proteinase K, or mock treated, as described (ELLIOTT *et al.*

2012). Protein samples to be analyzed by western blotting were separated on 12% SDS-PAGE gels and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore). Antibodies were polyclonal anti-Oxa1 generated against the whole protein (gift from J. Hermann), polyclonal anti- Cit1 (citrate synthase) and polyclonal anti-Yme1. Anti-rabbit IgG-HRP (BioRad) secondary antibodies were applied after washing and detected by the ECL detection method (Pierce).

To examine relative steady state levels of Oxa1 and Oxa1-T291K protein, total protein was prepared as described (Yaffe and Schatz 1984) from cells grown in CSM plus raffinose or CSM-luecine plus raffinose. Proteins were separated on 12% SDS-PAGE gels and transferred to Immobilon-FL polyvinlyidene fluoride membrane (Millipore). Antibodies were polyclonal anti-Oxa1 (gift from J. Hermann) and polyclonal anti- Cit1 (citrate synthase). Anti-rabbit Alexa Flour 488 IgG (Life Technologies) secondary antibodies were applied and detected using a FluorChem Q cabinet (AlphaImager).

Alignment of protein sequences and hydrophobicity plots

Protein sequences were aligned using ClustalW and boxshade via the ExPAsy portal (http://www.expasy.org/genomics/sequence_alignment). Hydrophobicity plots were generated by ExPAsy's ProtSscale (http://web.expasy.org/protscale/) using the Kyte and Doolittle method (KYTE and DOOLITTLE 1982).

Results

Substitution of charged residues for threonine 291 allows overexpressed Oxa1 to compensate for deletion of cox18

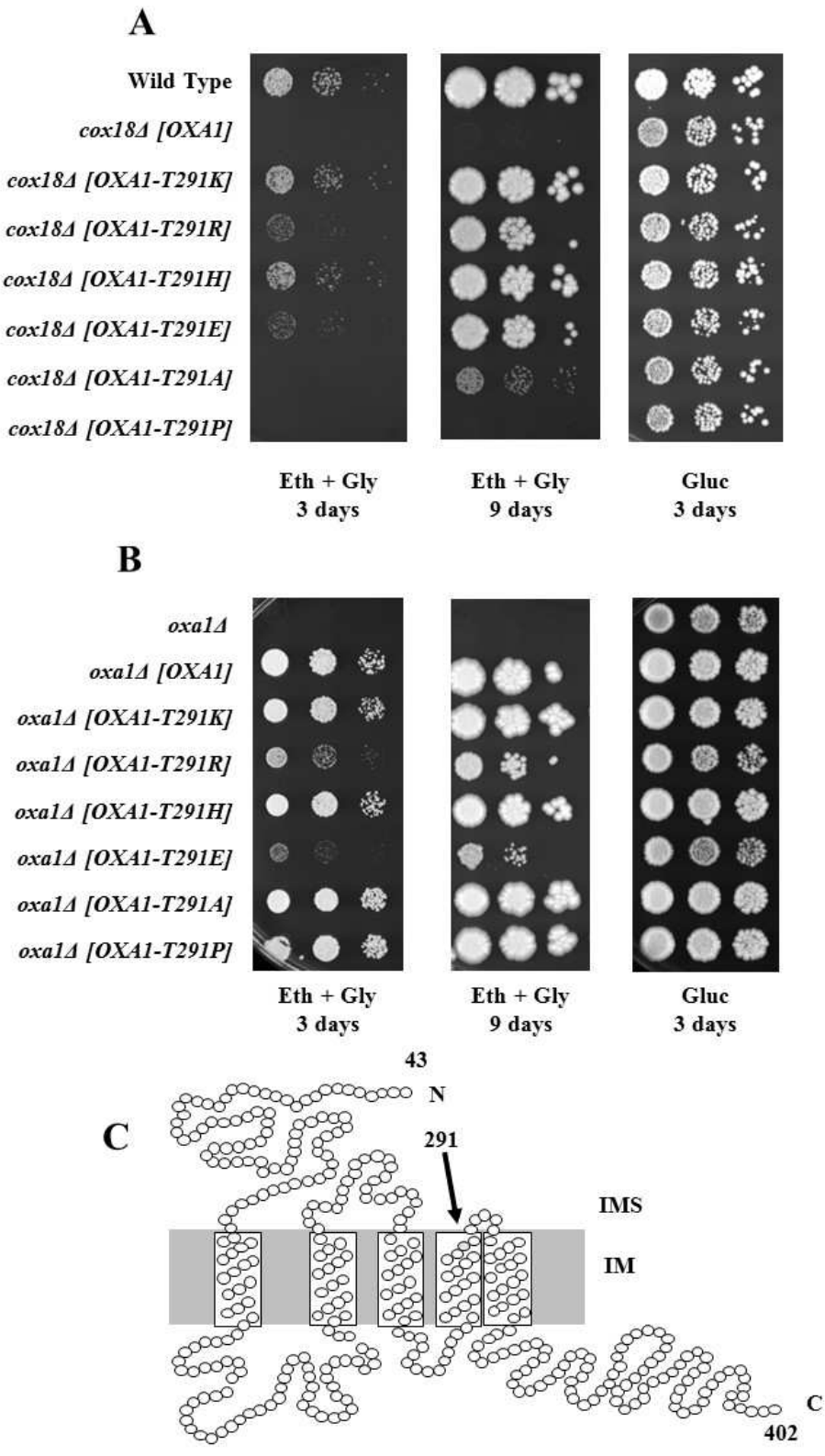
The respiring *cox18Δ* pseudorevertant 1c sup rho+ was initially isolated from the *cox18Δ [OXA1, LEU2]* strain SCS59 by Nathalie Bonnefoy. Respiration in this strain was shown to be linked to the presence of *LEU2* through plasmid loss experiments (Materials and Methods). The plasmid from this yeast strain was extracted and transformed into a naive *cox18Δ* yeast strain (Materials and Methods). The plasmid from 1c sup rho+ was sufficient to restore respiration to the *cox18Δ* strain. The *OXA1* insert from the plasmid was sequenced; the plasmid carried the *OXA1-T291K* allele. In order to identify more of these Oxa1 mutants, I created a *cox18Δ/Δ* diploid overexpressing *OXA1* from a high copy plasmid (LEE40). A diploid was used for this screen since a class of nuclear recessive mutations that allow for the bypass of *cox18* in strains overexpressing *OXA1* have already been isolated and studied (FIUMERA *et al.* 2009). Respiring pseudorevertants were rare; I only isolated three pseudorevertants from 11 independent cultures. The plasmids from these pseudorevertants were also extracted and sequenced. All three had plasmids with the *OXA1-T291R* allele.

Since changes to the T291 codon appeared to be important in bypassing *cox18*, I used site directed mutagenesis to introduce additional amino acid substitutions at T291 (Materials and Methods) and tested their ability to compensate for a deletion of *cox18*, when expressed from a 2 micron plasmid (Figure 3.1A). T291K, T291R,

T291H and T291E all allow for growth, although at varying levels, on ethanol/glycerol containing media in a *cox18Δ* background. Substitution of proline for threonine at position 291 initially appears to be unable to support growth, but after extended incubation there is growth on ethanol/glycerol media. Replacing threonine with alanine fails to allow growth of a *cox18Δ* strain on ethanol/glycerol media, even after 9 days incubation (Figure 3.1A).

Next I tested the ability of the mutant alleles to complement *oxa1Δ* by transforming an *oxa1Δ* strain with high copy plasmids carrying the various mutant alleles (Materials and Methods). All the alleles were able to restore some level of respiratory growth of an *oxa1Δ* mutant, containing a wild-type *COX18* gene (Figure 3.1B). The T291E allele grows the worst; all the other substitutions grow as well as wild type (Figure 3.1B). Therefore, a wide range of substitutions can be tolerated at position T291 without affecting respiratory growth. Only *OXA1-T291E* approaches a conversion of specificity, as it clearly compensates for a loss of *COX18* better than a loss of *OXA1*.

Figure 3.1: Mutations at position T291 in Oxa1 can compensate for respiratory growth in *cox18Δ* and *oxa1Δ* backgrounds when expressed from high copy plasmids. (A, B**)** Cells were grown in synthetic glucose medium or glucose medium lacking leucine, diluted and spotted onto YPAEG (*Materials and Methods*) nonfermentable ethanol plus glycerol medium (Eth + Gly), and incubated at 30°. This plate was photographed after 3 days and 9 days of incubation. Diluted cells were also spotted onto YPAD (*Materials and Methods*) glucose (Gluc), and incubated at 30° for 3 days. Strains are indicated as follows: Wild Type (DFS188), *cox18Δ* (CAB116), *cox18Δ [OXA1]* (LEE196), *cox18Δ [OXA1-T291K]* (LEE181), *cox18Δ [OXA1-T291R]* (LEE182), *cox18Δ [OXA1-T291H]* (LEE184), *cox18Δ [OXA1-T291E]* (LEE183), *cox18Δ [OXA1-T291P]* (LEE185), *cox18Δ [OXA1-T291A]* (LEE186), *oxa1Δ* (LEE41), *oxa1Δ [OXA1]* (LEE200), *oxa1Δ [OXA1-T291K]* (LEE201), *oxa1Δ [OXA1-T291R]* (LEE202), *oxa1Δ [OXA1-T291H]* (LEE204), *oxa1Δ [OXA1-T291E]* (LEE203), *oxa1Δ [OXA1-T291P]* (LEE205), *oxa1Δ [OXA1-T291A]* (LEE206) (C) Predicted topology of Oxa1 in the inner membrane (Lemaire, *et al* 2004) with residue 291 highlighted.


A
Wild Type
cox18Δ [OXA1]
cox18Δ [OXA1-T291K]
cox18Δ [OXA1-T291R]
cox18Δ [OXA1-T291H]
cox18Δ [OXA1-T291E]
cox18Δ [OXA1-T291A]
cox18Δ [OXA1-T291P]
Eth + Gly 3 days
Eth + Gly 9 days
Gluc 3 days
B
oxa1Δ
oxa1Δ [OXA1]
oxa1Δ [OXA1-T291K]
oxa1Δ [OXA1-T291R]
oxa1Δ [OXA1-T291H]
oxa1Δ [OXA1-T291E]
oxa1Δ [OXA1-T291A]
oxa1Δ [OXA1-T291P]
Eth + Gly 3 days
Eth + Gly 9 days
Gluc 3 days
C
43
N
291
IMS
IM
C
402

Oxa1-T291K steady-state protein levels are lower than Oxa1

OXA1-T291K was integrated from the endogenous *OXA1* locus (Materials and Methods) to determine its expression when expressed at normal levels from a single copy. When present at the chromosomal locus, *OXA1-T291K* supports wild-type levels of respiratory growth in an otherwise wild-type (*COX18*) background (Figure 3.2). In a *cox18* deletion background, *OXA1-T291K* allows respiratory growth, but more slowly than when *OXA1-T291K* is expressed from a high-copy plasmid in the same background (Figure 3.2). This indicates that Oxa1-T291K's ability to bypass *cox18* is not dependent on elevated gene expression and/or protein steady-state levels, but primarily is a result of the amino acid substitution.

Differences in protein steady state levels could account for the slower growth with the chromosomally integrated *OXA1-T291K*. I compared the relative steady-state amounts of Oxa1 and Oxa1-T291K protein expressed from the chromosomal locus and a 2 micron vector in the presence and absence of *COX18* (Figure 3.3). 50µg of total protein (Materials and Methods) from strains without plasmid or 5µg from strains bearing a plasmid were separated by SDS-PAGE on a 12% acrylamide gel. The resulting blot was probed for Oxa1 proteins and citrate synthase (as a loading control for mitochondrial proteins). In general, Oxa1-T291K levels are lower than Oxa1. Thus, the slower respiratory growth of *cox18 OXA-T291K* versus *cox18 oxa1Δ [OXA1-T291K]* might be the result of lower protein levels and less Oxa1-T291K activity.

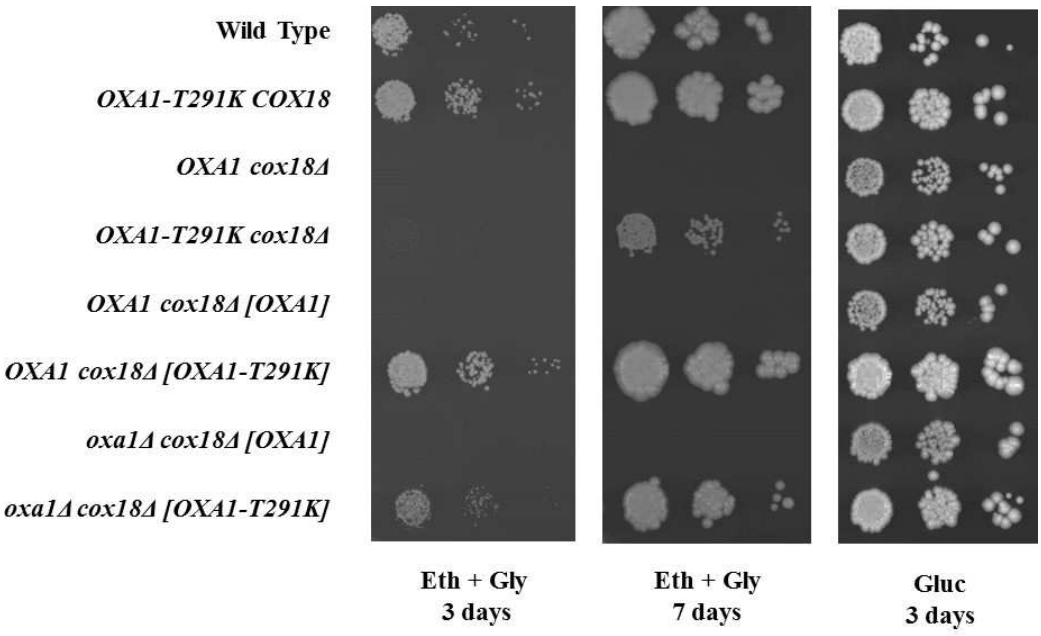


Figure 3.2: Oxa1-T291K can weakly suppress *cox18Δ* when expressed from the chromosomal locus. Cells were grown in synthetic glucose medium or glucose medium lacking leucine and diluted and spotted onto YPAEG (*Materials and Methods*) nonfermentable ethanol plus glycerol medium (Eth + Gly), incubated at 30° for 3 days and 7 days or spotted onto YPAD (*Materials and Methods*) glucose (Gluc), and incubated at 30° for 3 days. Strains are indicated as follows: Wild Type (DFS188), *OXA1-T291K COX18* (LEE44), *OXA1 cox18Δ* (CAB116), *OXA1-T291K cox18Δ* (LEE198), *OXA1 cox18Δ [OXA1]* (LEE196), *OXA1 cox18Δ [OXA1-T291K]* (LEE181), *oxa1 cox18Δ [OXA1]* (LEE199), *oxa1Δ cox18Δ [OXA1-T291K]* (LEE55).

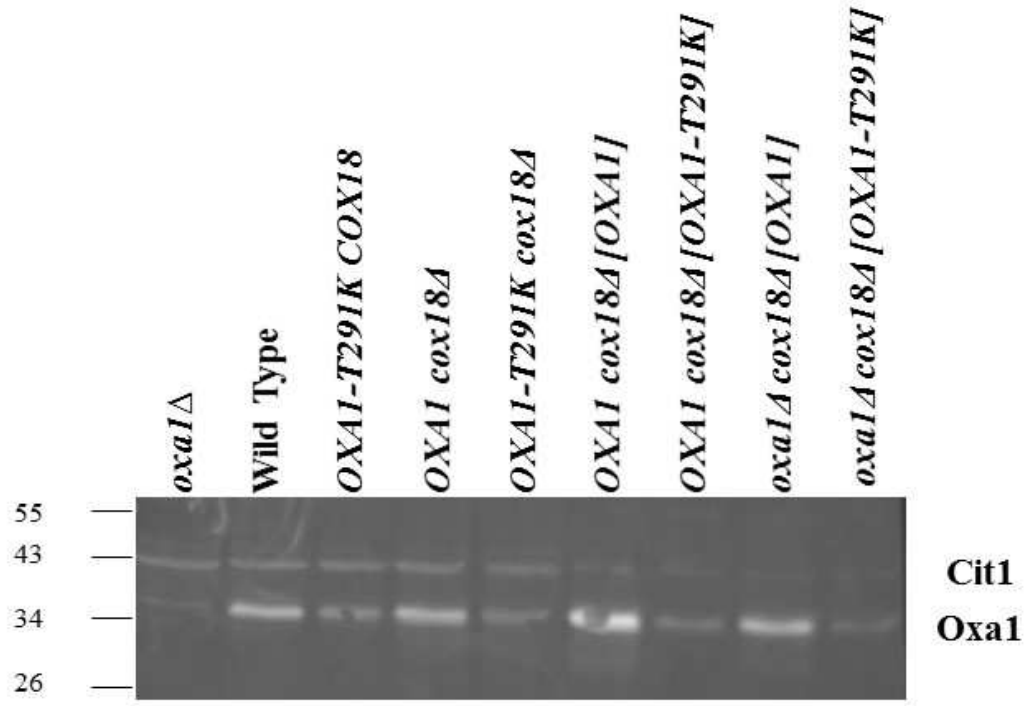


Figure 3.3: Relative steady state proteins levels of Oxa1 and Oxa1-T291K. Total cellular proteins (*Materials and Methods*) were separated on 12% SDS-PAGE and western blotted. For integrated strains, 50µg of proteins was loaded. For overexpression strains, 5µg of proteins was loaded. The blot was decorated with antibody specific against Oxa1 (Hermann) and antibody specific against Cit1, as a loading control. Strains are as follows: *oxa1Δ* (MCC319), Wild Type (DFS188), *OXA1-T291K COX18* (LEE44), *OXA1 cox18Δ* (CAB116), *OXA1-T291K cox18Δ* (LEE198), *oxa1 cox18Δ [OXA1]* (LEE199), *oxa1Δ cox18Δ [OXA1-T291K]* (LEE55).

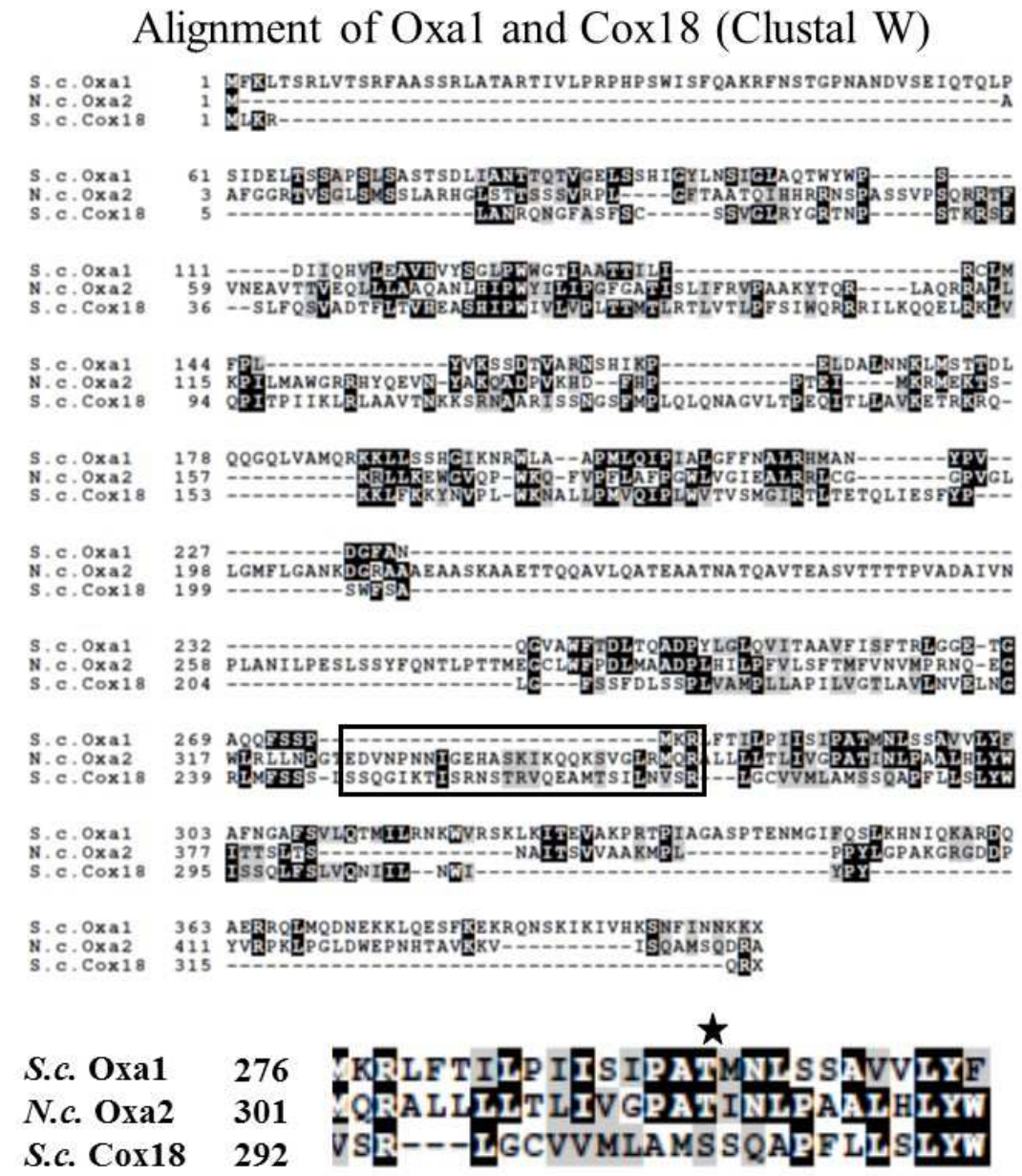
Residue T291 in Oxa1 is conserved across kingdoms and corresponds to S283 in Cox18

In order to understand how these substitutions in Oxa1 can compensate for loss of Cox18, I aligned the yeast protein sequences (Materials and Methods) to see if the substitutions made the Oxa1 sequence similar to the Cox18 sequence in that area. Although Oxa1 and Cox18 are paralogs and members of a protein family with functional conservation between kingdoms of life, they are highly divergent in primary sequence (YEN *et al.* 2001; BONNEFOY *et al.* 2009). In order to improve alignment between Oxa1 and Cox18 from *S. cerevisiae*, I included the Cox18 homolog, Oxa2, from *N. crassa* (FUNES *et al.* 2004b). Based on this alignment, position T291 of Oxa1 corresponds to S283 of Cox18 (Figure 3.4A). Therefore, the addition of large, charged residues does not make Oxa1 more closely resemble Cox18. The region around S283 of Cox18 lacks charged residues, in fact.

The broad range of allowable substitutions at residue 291 is surprising as Oxa1 homologs are fairly similar across kingdoms (Figure 3.4B). T291 (indicated by the star) from *S. cerevisiae* (yeast) is conserved in higher plants (*Arabidopsis*) and mammals (human), as are several surrounding residues. Despite this high conservation, threonine at this position is not absolutely required as all the substitutions tested retain some level of Oxa1 function in yeast (Figure 3.1B).

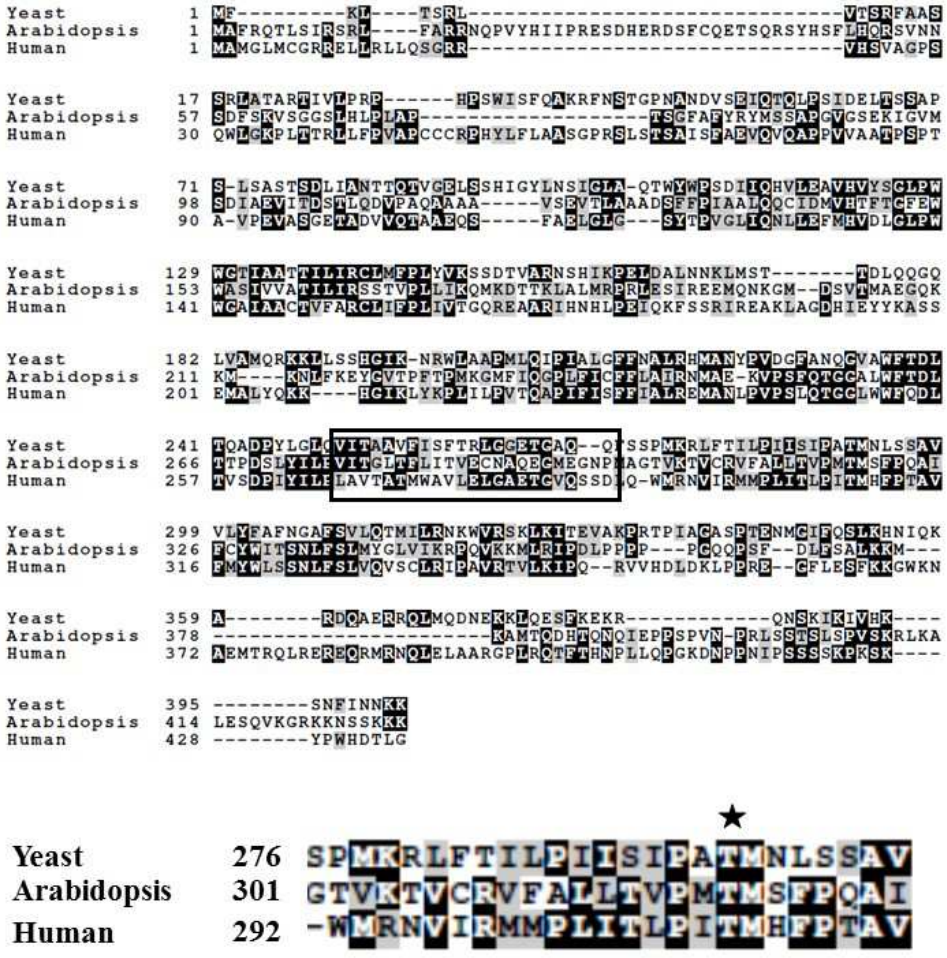
Figure 3.4: Alignment of Oxa1 to paralogs and homologs. (A) Alignment of Oxa1 and Cox18 from *S. cereviase* and Oxa2 sequences from *N. crassa*. Black shading indicates residues identical in at least two of the proteins while grey shading indicates similar residues in at least two of the proteins. Boxed and inset sequence is located around residue T291 of Oxa1. (B) Alignment of Oxa1 from *S. cerevisiae, A. thalania* and *H. sapien*. Black shading indicates residues identical in at least two of the proteins while grey shading indicates similar residues in at least two of the proteins. Boxed and inset sequence is located around residue T291 of Oxa1.

A


Alignment of Oxa1 and Cox18 (Clustal W)
S.c.Oxa1
N.c.Oxa2
S.c.Cox18
S.c. Oxa1 276
N.c. Oxa2 301
S.c. Cox18 292

B

Mutated threonine is conserved in Oxa1 homologs (Clustal W)



Oxa1 and Oxa1-T291K have no detectable differences in Proteinase K sensitivity

T291 is predicted to be at the end of the fourth transmembrane domain of Oxa1, near the intermembrane space (Figure 3.1C). The introduction of bulky and charged residues at this position could deform the surface of the protein facing the IMS, so I studied the topology of wild type and mutant Oxa1 proteins. Mitochondria were purified from *oxa1Δcox18Δ* strains expressing either Oxa1 or Oxa1-T291K from a high-copy plasmid and from wild type yeast and then converted to mitoplasts lacking the outer membrane (Materials and Methods). Both treated and untreated mitoplasts were solubilized, separated by SDS PAGE, and transferred to membrane (Materials and Methods). Oxa1 and its breakdown products were detected by western blotting with a polyclonal anti-Oxa1 antibody raised against the entire protein (personal communication from J. Herrmann). Without protease, both Oxa1 and Oxa1-T291K run at 40kDa (Figure 3.5). Protease digestion of mitoplasts from wild type yeast yeilded an approximately 27 kDa immunoreactive fragment. The Oxa1 antibody sometimes exhibits a cross-reacting band just above the 27 kDa fragment; it is likely reacting with something present outside the matrix, as treating mitoplasts with protease removes the band. This loss agrees with previous investigations and likely corresponds to the loss of the N-terminus, presumably down to the first transmembrane domain (HERRMANN *et al.* 1997). Protease digestion of Oxa1-T291K in mitoplasts from *oxa1Δ cox18Δ [OXA1-T291K]* also results in a 27kDa fragment.

There is no detectable difference between Oxa1 and Oxa1-T291k topology based on proteinase K sensitivity.

The blot was also probed with a polyclonal antibody against Yme1, a protein located in the IMS. This antibody produces a cross-reacting band that is resistant to protease degradation in mitoplasts but is lost in detergent treated samples treated with protease (Figure 3.5)(WILLIAMS *et al.* 2005). In mitoplasts that are treated with protease, Yme1 should be fully degraded. The Yme1 band was weakly seen in the *OXA1 COX18* sample, especially in mitochondria treated with protease. This could indicate leaky membranes in the mitochondria. Some protease clearly could pass the outer membrane in mitochondria in both strains, as indicated by the presence of some Oxa1 breakdown product in these samples. However Yme1 is clearly present in mitochondria, mitochondria plus protease and mitoplasts of *oxa1Δ cox18Δ [OXA1-T291K]*, but degraded in the mitoplasts plus protease and detergent treated plus protease samples.

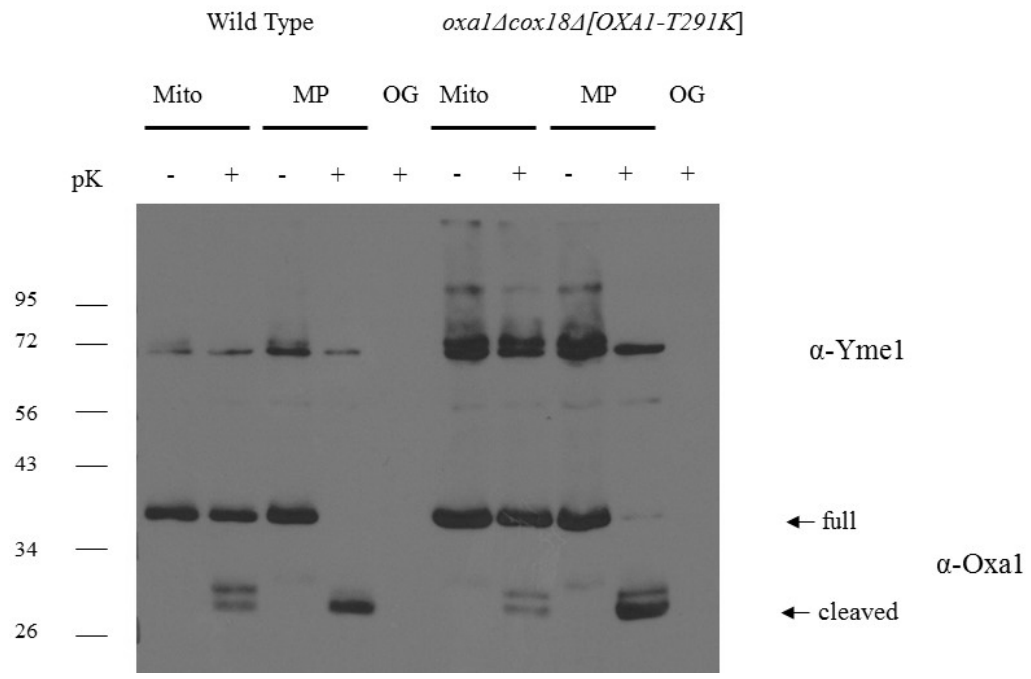


Figure 3.5: No detectable difference in protease K sensitivity of Oxa1 and Oxa1-T291K in mitoplasts. Mitochondria (Mito) and mitoplasts (MP) from strains expressing Oxa1 or overexpressing Oxa1-T291K were either mock treated or treated with 20μg/ml of proteinase K (pK) (*Materials and Methods*) as indicated. Samples were solubilized with 1% octyl glucoside (OG) prior to protease treatment where indicated. For *OXA1 COX18* and *oxa1Δ cox18[OXA1-T291K]*, 20 μg of proteins were loaded per lane; the resulting blots were probed with α-Oxa1 and α-Yme1 (inter membrane space marker) antibodies. Blots were developed with the ECL detection system (Pierce). Strains are indicated as follows: Wild Type (DFS188), and *oxa1 cox18Δ [OXA1-T291K]* (LEE55).

Oxa1-T291K bypasses known partners of Cox18

In order to better understand how Oxa1-T291K and similar substitutions are able to bypass the need for Cox18 in the assembly of Cox2, I tested the ability of the T291K allele to support respiration in the absence of other factors known to participate in Cox2 C-tail export together with Cox18. Cox18 forms a complex with two other proteins, Mss2 and Pnt1 (SARACCO and FOX 2002). A deletion of *mss2* results in a respiratory negative phenotype due to failure of Cox2 C-tail export (BROADLEY *et al.* 2001). A *pnt1* deletion does not impair respiratory growth of otherwise wild-type *S. cerevisiae*, although it appears to participate in Cox2 C-tail export (HE and FOX 1999). *OXA1-T291K*, when expressed from a high copy plasmid, is able to restore respiration to a *mss2Δ* strain, but high copy expression of *OXA1* cannot (Figure 3.6). Thus Oxa1-T291K does not simply replace Cox18 in this complex.

I recently showed that Cox18 physically interacts with the non-canonical chaperone Cox20 (ELLIOTT *et al.* 2012). Cox20 is additionally required for the N-terminal processing of Cox2 (HELL *et al.* 2000; ELLIOTT *et al.* 2012). Overexpression of *OXA1-T291K* is capable of weakly restoring respiration to a *cox20Δ* strain, and a *cox20Δ cox18Δ* strain (Figure 3.7). Respiratory growth in the absence of *cox20* is stronger when *COX18* is present in addition to overexpression of *OXA1-T291K*. I also showed that *cox20Δ* can by bypassed by disrupting the Yme1 protease, which is responsible for degrading unassembled Cox2 peptide that is present in the IMS (ELLIOTT *et al.* 2012). Mgr1 is an accessory protein to the Yme1 protease and a *mgr1Δ cox20Δ* strain processes the N-terminus of Cox2, although inefficiently, and

weakly respires (ELLIOTT *et al.* 2012). I proposed that this bypass system works by stabilizing the inserted, but not yet assembled Cox2 peptide, allowing for some processing and assembly into cytochrome *c* oxidase. It is possible that Oxa1-T291K is also acting to stabilize Cox2. I constructed a *cox20Δ cox18Δ mgr1Δ* triple mutant strain, which does not respire (Figure 3.7B). Overexpression of Oxa1-T291K is able to restore respiration and in fact respires better than either the *mgr1Δ cox20Δ* or the *cox20Δ cox18Δ [OXA1-T291K]* strains (Figure 3.7B). Suppression of *cox18Δ* by overexpressed *OXA1* and deletion of *mgr1* or *mgr3* required functional Yme1 (FIUMERA *et al.* 2009). Oxa1-T291K, in contrast, is able to restore respiration in a *cox18Δ yme1Δ* strain (data not shown). Thus Oxa1-T291K bypass of *cox18Δ* is independent of Yme1, unlike the previously reported *cox18Δ* suppressors. Additionally, Oxa1-T291K does not require *mgr1* or *mgr3* to bypass *cox18Δ* (data not shown).

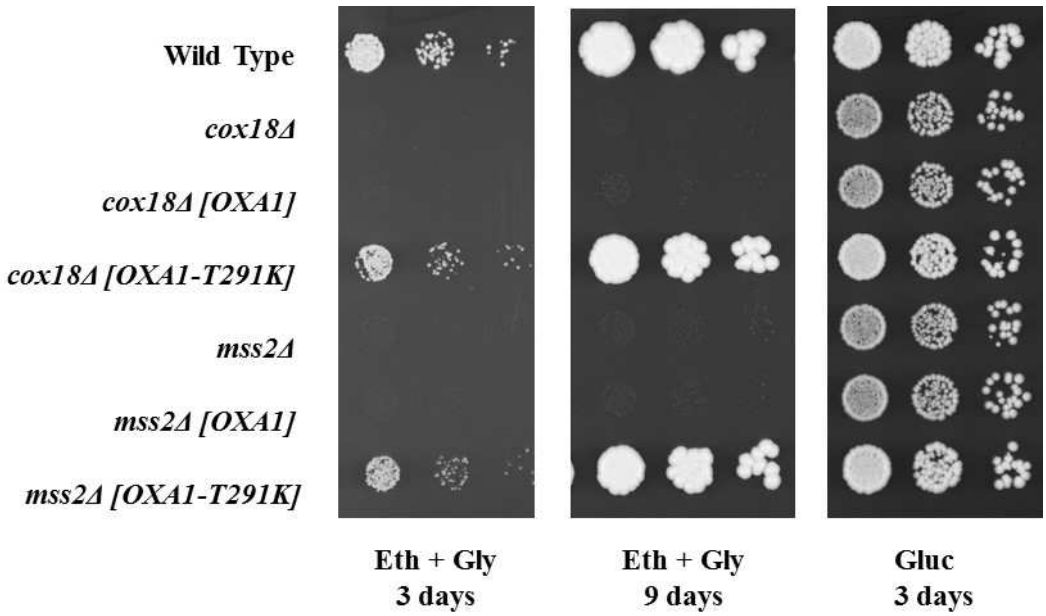


Figure 3.6: Overexpressed Oxa1-T291K bypasses Mss2 as well as Cox18, by a pathway that does not require Yme1, Mgr1 and Mgr3. Cells were grown in synthetic glucose medium or glucose medium lacking leucine and diluted and spotted onto YPAEG (*Materials and Methods*) nonfermentable ethanol plus glycerol medium (Eth + Gly), incubated at 30°. This plate was photographed after 3 days and 9 days of incubation. Diluted cells were also spotted onto YPAD (*Materials and Methods*) glucose (Gluc), and incubated at 30° for 3 days. Strains are indicated as follows: Wild Type (DFS188), *cox18Δ* (CAB116), *cox18Δ [OXA1]* (LEE196), *cox18Δ [OXA1-T291K]* (LEE181), *mss2Δ* (XPM184a), *mss2Δ [OXA1]* (LEE210), *mss2 [OXA1-T291K]* (LEE47).

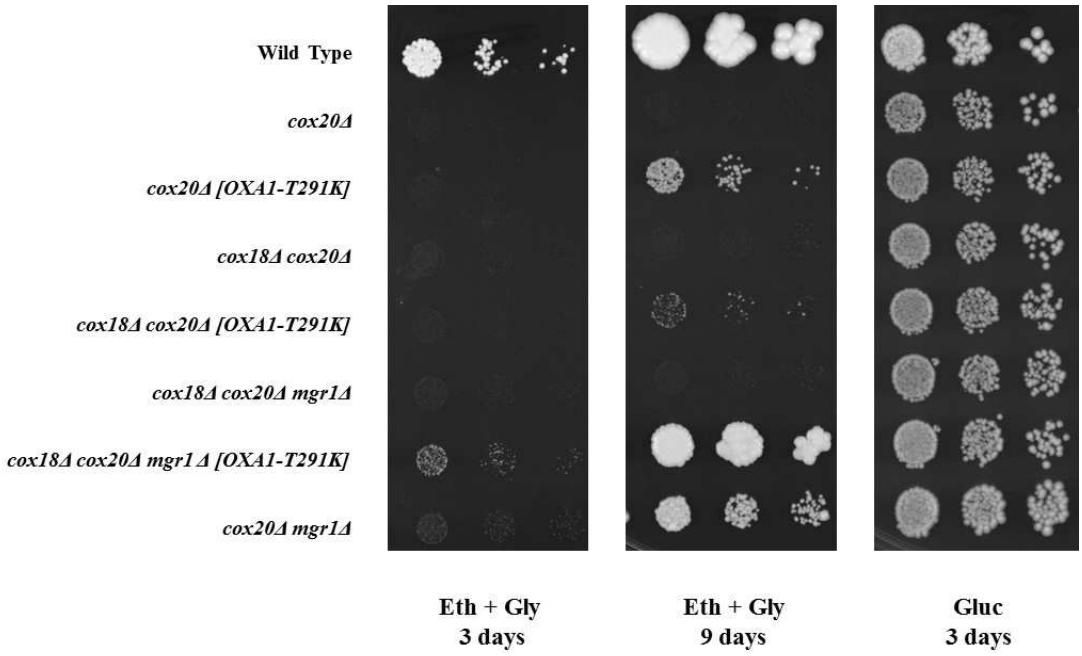


Figure 3.7: Overexpressed Oxa1-T291K weakly bypasses Cox20. Cells were grown in synthetic glucose medium or glucose medium lacking leucine and diluted and spotted onto YPAEG (*Materials and Methods*) nonfermentable ethanol plus glycerol medium (Eth + Gly), incubated at 30° for 3 days and 9 days or spotted onto YPAD (*Materials and Methods*) glucose (Gluc), and incubated at 30° for 3 days. Strains are indicated as follows: Wild Type (DFS188), *cox20Δ* (SCS194), *cox20Δ [OXA1-T291K]* (LEE156), *cox1Δ8 cox20Δ* (LEE81), *cox18Δ cox20Δ [OXA1-T291K]* (LEE96), *cox18Δ cox20Δ mgr1Δ* (LEE171A), *cox18Δ cox20Δ mgr1Δ [OXA1-T291K]* (LEE172A) and *cox20Δ mgr1Δ* (LEE99).

Discussion

The data presented here clearly show that with a single amino acid substitution, Oxa1 can gain the ability to replace Cox18 in the assembly of Cox2 into cytochrome *c* oxidase. When expressed at high levels, Oxa1 is capable of inserting both termini of Cox2 through the inner membrane; however, this fully-inserted Cox2 is incompetent for assembly, as *cox18Δ [OXA1]* fails to respire (FIUMERA *et al.* 2009). Substitution of large, charged amino acids (arginine, lysine, histidine, and glutamic acid) for threonine at position 291 of Oxa1 allow for robust respiratory growth of *cox18Δ* cells, while substitution of proline allowed for weak growth (Figure 3.1A). Presumably these alleles have gained the ability to assemble Cox2. Since these mutations still have their C-terminal ribosome interaction domains, it is clear that this domain does not completely preclude Cox18 function as previously proposed (PREUSS *et al.* 2005).

This is the first identification of mutations in one of this pair of paralogs that are able to directly compensate for the other. Both Oxa1 and Cox18 can be bypassed by disrupting other pathways. The only other known Cox18 bypass requires the overexpression of Oxa1 in the absence of either of the *i*-AAA protease adaptors, Mgr1 and Mgr3 (FIUMERA *et al.* 2009). Conversely, Oxa1 has several known bypass mechanisms. Mutating alanine 189 of Cox2 to proline was reported to allow for bypass of an *oxa1* temperature sensitive allele, and assembly of Cox2 without N-terminal processing (MEYER *et al.* 1997a). Unfortunately, no further study of this interesting Cox2 allele has occurred. A pair of substitutions in the C-terminal membrane anchor of cytochrome *c1* (Cyt1) protein restore both cytochrome *c* oxidase

and ATPase activity in an *oxa1* temperature sensitive yeast strain (HAMEL *et al.* 1998). High expression of the yeast methyltransferase-like protein Oms1 is able to compensate for at least three mutant alleles of *oxa1*, although it cannot suppress a full deletion (LEMAIRE *et al.* 2004).

Oxa1-T291K retains all normal Oxa1 function, since *OXA1-T291K COX18* respires as well as *OXA1 COX18* (Figure 3.2). In addition to fulfilling the normal functions, *OXA1-T291K* can restore respiration to *cox18Δ* both at single copy and when overexpressed (Figure 3.2). Two possible mechanisms are readily apparent: A) Oxa1-T291K is directly replacing Cox18 in an interaction with another factor, likely an assembly factor or B) Oxa1-T291K interacts with the Cox2 peptide in a novel way that allows for Cox2 to enter its normal assembly pathway.

To address the first possibility, I compared the sequence of Oxa1 and Cox18 in order to determine which residue in Cox18 aligned with T291 in Oxa1 (Figure 3.4A). The corresponding residue is S283. None of the Oxa1 substitutions that complement *cox18Δ* are biochemically similar to serine; therefore I conclude that the mutant Oxa1 proteins are not becoming more like Cox18 at the level of primary sequence.

T291 is predicted to be at the very end of transmembrane (TM) 4 of Oxa1, close to the intermembrane space (Figure 3.1C). Substitution of bulky, charged residues (lysine, arginine, histidine and glutamic acid) all allow respiratory growth in a *cox18Δ* strain (Figure 3.1A). Additionally, substitution of proline also allows for respiratory growth, but at a much slower rate (Figure 3.1A). Clearly, the presence of a charge increases the efficiency at which the mutant Oxa1 protein assembles Cox2.

Proline is known to disrupt alpha helixes (although usually when it is in the middle of the structure). Therefore it is possible that all these residues are excluded from the membrane and actually reside in the intermembrane space. Attempts to visualize difference in topology in mitoplasts treated with protease failed to show any difference in cleavage of Oxa1 or Oxa1-T291K (Figure 3.4). Loop 4 between TM4 and TM5 is predicted to be very small and to date no cleavage in this loop has ever been seen in native Oxa1 proteins (HERRMANN *et al.* 1997; SATO and MIHARA 2009). Therefore this lack of novel cleavage does not necessarily rule out a change in topology, but might rather be the result of protease being unable to access this region of the protein.

Although the crystal structure of Oxa1 has not been solved, a cryo-EM structure of Oxa1 in complex with the mitochondrial ribosome has been published (KOHLER *et al.* 2009). Based on this structure, Oxa1 appears to exist as a homodimer situated over the ribosomal exit tunnel. TM4 is thought to be on the periphery of the Oxa1 dimer and not situated at the insertion pore. Analysis of intragenic suppressors of mutations spread throughout the Oxa1 protein suggest that TM4 has functional interactions with TM5 and loop2 (between TM3 and TM4) (MATHIEU *et al.* 2010). This placement of residue in the 3-dimensional conformation of the Oxa1 dimer seems more likely to cause a change in interactions with other molecules, rather than affecting movement of substrate through the insertion pore.

I tested Oxa1-T291K's requirement for known Cox18 partners; Cox18 is known to physically interact with three partners, in addition to its interaction with the Cox2 peptide: Mss2, Pnt1 and Cox20 (SARACCO and FOX 2002; ELLIOTT *et al.* 2012).

Cox18 forms a complex with Mss2 and Pnt1 on the matrix side of the inner membrane (SARACCO and FOX 2002). Mss2 is essential for respiratory growth, but Pnt1 is not (HE and FOX 1999; BROADLEY *et al.* 2001). Since Oxa1-T291K is sufficient to restore respiration in *mss2Δ cox18Δ* (Figure 3.6), I conclude that Oxa1-T91K does not require Mss2 or Pnt1, and is not replacing Cox18 in this complex.

Cox20 is essential for respiratory growth and has two known roles in the genesis of Cox2: efficient processing of the Cox2 peptide by the Imp1 protease and stabilizing the Cox2 peptide after it has been fully inserted into the inner membrane, but not yet assembled into cytochrome *c* oxidase (HELL *et al.* 2000; ELLIOTT *et al.* 2012). Cox20 and Cox18 most likely interact in the intermembrane space and their interaction is dependent on the presence of the Cox2 peptide (HELL *et al.* 2000; ELLIOTT *et al.* 2012). Oxa1-T291K is capable of bypassing a *cox20Δ*, when it is expressed from a high copy plasmid (Figure 3.7). However, respiratory growth is quite slow in either *cox20Δ [OXA1-T291K]* or *cox20Δ cox18 [OXA1-T291K]*. From previous work, I know that *cox20* can also be bypassed by deleting components of the *i*-AAA protease (Yme1, Mgr1 and Mgr3) resulting in slow N-terminal processing of Cox2 and very weak respiratory growth (ELLIOTT *et al.* 2012). When I combined a *mgr1* deletion with *cox18Δ cox20Δ [OXA1-T291K]*, respiratory growth was increased over the growth of either bypass alone (Figure 3.7). I previously proposed that the processing and growth seen in the absence of *cox20* are the result of increased Cox2 stability. The growth of *cox18Δ cox20Δ mgr1Δ [OXA1-T291K]* suggests that Oxa1-T291K may be operating in a similar manner and is bypassing known Cox18 partners, such as Cox20, by stabilizing the fully inserted but unassembled Cox2 peptide.

Deletions in *MGR1* and *MGR3* are also able to bypass the need for Cox18 in strains overexpressing *OXA1* (FIUMERA *et al.* 2009). Bypass of *cox18Δ* by overexpressed *OXA1* requires Yme1 to act in as chaperone (FIUMERA *et al.* 2009). Bypass of *cox18Δ* by overexpressed *OXA1-T291K*, however, occurs even in the absence of *yme1* (Figure 3.6). This difference suggests that Oxa1-T291K is bypassing *cox18Δ* in a way that is distinct from how overexpression of *OXA1* bypasses *cox18Δ* in the absence of Mgr1 or Mgr3, pointing to a novel mechanism for bypassing *cox18Δ* by Oxa1-T291K, one that does not require the chaperone activity of Yme1.

Since Oxa1-T291K does not require any factors known to interact with Cox18, I conclude that Oxa1-T291K is not simply replacing Cox18 in its usual complexes or roles. It is still possible that Oxa1-T291K is interacting with another factor to promote the assembly of Cox2 after its insertion into the inner membrane. However, it is just as likely that Oxa1-T291K is promoting assembly of Cox2 into cytochrome oxidase through a changed interaction with the Cox2 peptide itself. Based on the ability of bypass *cox20Δ*, it seems likely that Oxa1-T291K (and the other mutant alleles) are stabilizing the Cox2 peptide after both termini are exported into the IMS.

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